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(54) Title: ABL-INTERACTOR PROTEIN (57) Abstract The present invention relates to a protein that interacts with the cAbl protein tyrosine kinase and to a nucleic acid sequence encoding same. The invention also relates to complexes of the protein of the invention and cAbl and to the use of such complexes in the identification of therapeutic and diagnostic agents.		

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ABL-INTERACTOR PROTEIN

This is a continuation-in-part of Application No. 08/549,004, filed October 27, 1995, the entire contents of which are hereby incorporated by
5 reference.

This application was made with Government support under Grant No. CA 61033 awarded by the National Institutes of Health. The Government has certain rights in the invention.

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TECHNICAL FIELD

The present invention relates to a protein that interacts with the cAbl protein tyrosine kinase and to a nucleic acid sequence encoding same. The invention also relates to complexes of the protein of
15 the invention and cAbl and to the use of such complexes in the identification of therapeutic and diagnostic agents.

BACKGROUND

The c-Abl protein, originally identified as the cellular homolog of the v-abl oncogene product of Abelson murine leukemia virus (A-MuLV) (Goff et al, Cell 22:777 (1980); Wang et al, Cell 36:349 (1984)), is a tyrosine kinase of unknown function. Biochemical data suggest that c-Abl may regulate signal transduction events in the cytoplasm and processes in the nucleus. c-Abl is found primarily in the nucleus (Van Etten et al, Cell 58:669 (1989)), but it is also found in association with the plasma membrane and bound to actin filaments in the cytoplasm (Van Etten et al, Cell 58:669 (1989)); Van Etten et al, J. Cell. Biol. 124:325 (1994)).

The c-Abl protein has a complex structure that includes several domains common to proteins implicated in signal transduction pathways. Among these domains are the non-catalytic Src homology 2 and 3 (SH2 and SH3) domains and the tyrosine kinase (SH1) domain. SH2 and SH3 domains are modular components present in a large number of proteins (Pawson, Nature 373:573 (1995)). These domains are critical in the formation of stable signaling protein complexes, and have also been shown to regulate protein function (Feller et al, Trends Biochem. Sci. 19:453 (1994); Cohen et al, Cell 80:237 (1995); Pawson, Nature 373:573 (1995)). The SH3 domain

suppresses the intrinsic transforming activity of c-Abl *in vivo* (Franz et al, EMBO J. 8:137 (1989); Jackson and Baltimore, EMBO J. 8:449-456 (1989)), while the SH2 domain is required for the transforming function of activated *abl* genes (Mayer et al, Mol. Cell. Biol. 12:609 (1992); Mayer and Baltimore, Mol. Cell. Bio. 14:2883 (1994)). The unique carboxy(C)-terminal region of c-Abl, which is encoded by a single exon, contains several functional and structural domains that include a nuclear localization signal (Van Etten et al, Cell 58:669 (1989)), proline-rich sequences that have the potential to bind to SH3-domain-containing proteins (Feller et al, EMBO J. 13:2341 (1994); Feller et al, Trends Biochem. Sci. 19:453 (1994); Ren et al, Genes & Dev. 8:783 (1994)), a DNA-binding domain (Kipreos and Wang, Science 256:382 (1992)) and an actin-binding domain (Van Etten et al, J. Cell. Biol. 124:325 (1994); McWhirter and Wang, EMBO J. 12:1533 (1993)). Several serine/threonine residues within the C-terminal exon are phosphorylated by the cdc 2 kinase (Kipreos and Wang, Science 248:217 (1990)) and by protein kinase C (Pendergast et al, Mol. Cell. Biol. 7:4280 (1987)). The presence of multiple structural and functional domains within the c-Abl tyrosine kinase and its localization to cytoplasmic and nuclear cellular compartments, suggest a potential role for c-Abl in the regulation of

transcription, DNA replication or cell cycle progression, as well as in the control of signaling events in the cytoplasm.

The tyrosine kinase activity of c-Abl is tightly regulated in vivo (Pendergast et al, Proc. Natl. Acad. Sci. USA 88:5927 (1991); Mayer and Baltimore, Mol. Cell. Bio. 14:2883 (1994)). Overexpression of c-Abl at levels 5- to 10- fold over the endogenous c-Abl protein does not lead to cell transformation but causes growth arrest (Jackson and Baltimore, EMBO J. 8:449 (1989); Jackson et al, EMBO J. 12:2809 (1993); Sawyers et al, Cell 77:121 (1994)). In contrast, structurally altered forms of Abl cause cell transformation and exhibit elevated tyrosine kinase activity when expressed at similar levels (Franz et al, EMBO J. 8:137 (1989); Jackson and Baltimore, EMBO J. 8:449 (1989); Muller et al, Mol. Cell. Biol. 11:1785 (1991)).

Activation of the oncogenic potential of c-Abl has been shown to occur as a consequence of structural alterations in the amino(N)- or C-terminal sequences (reviewed in Wang, Curr. Opin. Genet. Dev. 3:35-43 (1993)). Three naturally occurring c-abl-derived oncogenes have been identified (Goff et al, Cell 22:777 (1980); Bergold et al, J. Virol. 61:1193 (1987); Pendergast and Witte, In: Balliere's Clinical Haematology 1(4):1001 (1987); Kurzrock et al, N. Engl. J. Med. 319:990 (1988)). Oncogenic activity

has been shown to result from, or be associated with, deletion of the Abl SH3 domain and fusion with *gag* sequences following retroviral transduction (Franz et al, EMBO J. 8:137 (1989); Jackson and Baltimore, EMBO J. 8:449 (1989); Muller et al, Mol. Cell. Biol. 11:1785 (1991)), deletion of Abl C-terminal sequences and fusion with viral sequences, while retaining the Abl SH3 domain (Bergold et al, J. Virol. 61:1193-1202 (1987)), and fusion of *bcr* sequences upstream of the second exon of *c-abl* (Muller et al, Mol. Cell. Biol. 11:1785 (1991); McWhirter and Wang, Mol. Cell. Biol. 11:1553 (1991); Pendergast et al, Cell 66:161 (1991)). Mutants of *c-Abl* have also been generated experimentally that exhibit increased transforming activity. These include Abl proteins with deletions or alterations in the SH3 and C-terminal sequences (Franz et al, EMBO J. 8:137 (1989); Jackson and Baltimore, EMBO J. 8:449 (1989); Goga et al, Mol. Cell. Biol 13:4967 (1993); Mayer and Baltimore, Mol. Cell. Biol. 14:2883 (1994)). The structural alterations in the mutated Abl proteins disrupt the negative regulatory mechanisms that control the *c-Abl* protein tyrosine kinase, generating transforming Abl proteins that are constitutively active and are primarily localized in the cytoplasm.

Several possible mechanisms have been suggested for the inhibition of the *c-Abl* tyrosine kinase. Recently, it has been shown that the inhibitory

effect of the Abl SH3 domain is extremely position sensitive (Mayer and Baltimore, Mol. Cell. Biol. 14:2883 (1994)). These results suggest that, in addition to the SH3 domain, other region(s) of c-Abl may be required for repression. Two potential mechanisms have been proposed. First, it is possible that the SH3 domain functions in cis by binding to another region of Abl and effectively locking the protein in an inactive conformation. A second model consistent with the available data suggests that the c-Abl protein is negatively regulated by a trans-acting cellular modulator that exerts its effects by interacting with the Abl SH3 domain and a second region of the Abl protein (Mayer and Baltimore, Mol. Cell. Bio. 14:2883-2894 (1994)). The present invention provides such a protein.

SUMMARY OF THE INVENTION

The present invention relates to a protein that interacts with both the SH3 domain and carboxy terminal sequences of the cAbl tyrosine kinase (ie, it is an Abl interactor (Abi) protein). The present protein is a substrate for the cAbl tyrosine kinase. The protein of the invention contains a SH3 domain and proline-rich sequences critical for binding to cAbl. A basic region in the amino-terminus of the protein of the invention is homologous to the DNA-

binding sequence of homeodomain proteins. The properties of the Abi protein indicate that the protein has a dual role as a regulator and effector of the cAbl protein and that the Abi protein functions as a tumor suppressor in cells.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. Sequence and Northern Blot Analysis of Abi-2

(A) Predicted amino acid sequence of human Abi-2. Sequences in the C terminus corresponding to the SH3 domain are boxed. The three potential SH3 binding sites are in bold and a serine-rich region is in bold and underlined. A potential c-Abl tyrosine phosphorylation site is doubly underlined and the tyrosine is marked with an asterisk. Three PEST regions are bracketed with arrows and a homeodomain homologous region at the N-terminus is underlined. A polyproline stretch is underlined with dashed lines. (B) Diagram of structural features of Abi-2. (C) Alignment of the Abi-2 homeodomain homologous region with several homeodomains. The consensus sequence is shown at the bottom. (D) Northern blot analysis of *abi-2* expression in human tissues. Two micrograms of poly A⁺ RNA from the indicated human tissues were hybridized to ³²P-labeled full length *abi-2* cDNA probe. The RNA markers are indicated at the left.

Figure 2. *Abi-2* encoding sequence. The ATG start and TGA stop codons are marked, as are a potential alternative splice site and the poly A site.

Figure 3. Expression and subcellular localization of *Abi-2* proteins

(A) *In vitro* transcription and translation of *abi-2* cDNA. *In vitro* transcription/translation was performed without DNA (lane 1), with pGEM vector alone (lane 2), or with pGEM/*abi-2* (lane 3). ³⁵S methionine labeled protein products were separated on SDS-PAGE directly (lanes 1 to 3) or incubated with preimmune serum (lane 4) or anti-*Abi-2* serum (lane 5) and the complexes collected on Protein A Sepharose prior to SDS-PAGE separation. Labeled proteins were visualized by fluorography. (B) Expression of *Abi-2* *in vivo*. Bosc 23 cells were transfected with pCGN vector alone (lane 1), or pCGN/*abi-2*, (lanes 2 and 3). Total cell lysates were separated on SDS-PAGE and subjected to Western blot analysis with an anti-HA monoclonal antibody (lane 2) or anti-*Abi-2* antibody (lane 3). For analysis of endogenous *Abi-2* expression, lysates from human RAMOS cells (2×10^5 cells) were subjected to SDS-PAGE followed by Western blot analysis with anti-*Abi-2* antibody (lane 4). The *Abi-2* proteins are marked with the arrows. (C) Subcellular localization of *Abi-2*. Approximately 2×10^7 RAMOS cells were swollen in hypotonic buffer for

10 min., and subcellular fractions were prepared. Cell lysates were centrifuged to separate the nuclei from the cytosol/membrane fraction. Equal amounts of total cell lysate, cytosol/membrane, and nuclei were
5 analyzed on an 8% SDS-PAGE, followed by Western blotting with anti-Abi-2 antibodies. The blot was developed with the ECL detection kit.

Figure 4. Abi-2 interacts with c-Abl

(A) Sf9 insect cells were infected with either
10 c-Abl or GAP recombinant baculoviruses and labeled with ^{35}S methionine. Cell lysates were incubated with equal amounts of immobilized GST alone (lanes 1 and 4), GST-Abi-2 (lanes 2 and 5), and anti-Abl (lane 3) or anti-GAP (lane 6) antibodies bound to protein
15 A-Sepharose beads. After incubation for 90 min. at 4°C, the beads were washed with RIPA buffer to remove unbound material. Bound proteins were separated on SDS-PAGE and visualized by fluorography. The positions of c-Abl and GAP are indicated. (B) Bosc
20 32 cells co-transfected with the pSR α /c-abl and pCGN/abi-2 expression vectors were lysed and immunoprecipitated with preimmune serum (lane 1), anti-Abi-2 (lane 2), and anti-Abl antibodies (lane 3). The immunoprecipitated proteins were
25 subjected to an *in vitro* kinase assay in the presence of [γ - ^{32}P]ATP. The phosphorylated proteins were separated on SDS-PAGE and visualized by

autoradiography. (C) Following immunoprecipitation (I.P.) and kinase assay as described in B, the bound proteins were eluted from the immune complexes by boiling in the presence of SDS-PAGE sample buffer and subjected to a second cycle I.P. with the indicated sera. Proteins immunoprecipitated by the second cycle I.P. were separated on SDS-PAGE and visualized by autoradiography.

Figure 5. Abi-2 binds to c-Abl at both SH3 and C-terminal regions

(A) Interaction of Abi-2 with c-Abl is retained following deletion of the c-Abl SH3 domain. Lysates of Cos cells transfected with expression vectors encoding wild type c-Abl (pSR α /c-abl) (lanes 1-3) or an SH3 deletion mutant of c-Abl (pSR α /c-abl Δ SH3) (lanes 4-6) were incubated with immobilized GST alone (lanes 1, 4), GST-Abi-2 (lanes 2, 5), or anti-Abl antibodies bound to Protein A-Sepharose beads (lanes 3, 6). Bound proteins were separated on SDS-PAGE, transferred to nitrocellulose, and immunoblotted with anti-Abl antibodies. Proteins were visualized as described in Figure 3. (B) Mapping of the Abi-2 binding sites on c-Abl. Wild type c-Abl (top) and c-Abl deletion mutants are schematically shown. Binding of c-Abl fragments to Abi-2 was determined either by the yeast two-hybrid system assay or by an *in vitro* binding assay, as indicated to the right.

NLS: nuclear localization sequence; BD: binding domain. (C) Deletion of the Abl SH3 domain and Abl-C-terminal proline-rich sequences abolishes Abl binding to Abi-2. The indicated *in vitro* translated proteins were labeled with ^{35}S methionine and incubated with GST alone, GST Abi-2, and anti-Abl antibodies bound to Protein A-Sepharose beads, as indicated. After incubation for 60 min. at 4°C , the complexes were washed and bound proteins were analyzed by SDS-PAGE. ^{35}S labeled proteins were visualized by fluorography. (D) The binding of Abi-2 to the Abl SH3 domain is specific. *abi-2* cDNA was subcloned into pAS1-CYH2 and expressed in yeast as a GAL4 DB-fusion which contains the influenza hemagglutinin (HA) epitope tag. Yeast cell lysates were incubated with GST or GST-fusion proteins as indicated. Bound proteins were separated on a 10% SDS-PAGE, transferred to nitrocellulose, and analyzed by Western blotting using anti-HA antibody. The arrow indicates the HA-tagged Abi-2 protein.

Figure 6. c-Abl binds to the proline-rich N-terminus and the C-terminal SH3 domain of Abi-2

(A) Schematic representation of the Abi-2 deletion mutants constructed. (B) cDNAs encoding *abi-2*, *abi-2 Δ SH3*, and *abi-2 Δ 244-401* were subcloned into the pGEM vector for *in vitro* transcription/translation. The ^{35}S methionine

translated proteins were incubated with anti-Abi-2 antibodies bound to Protein A (lanes 1, 4 and 7), GST-Abl SH3 (lanes 2, 5 and 8), and GST-Abl (593-730) (lanes 3 and 6). Bound proteins were washed and
5 separated on SDS-PAGE and ³⁵S labeled proteins were visualized by fluorography. (C) To map the region in Abi-2 that binds to Abl SH3 domain, cDNAs encoding N-terminal deletion mutants (*abi-2Δ1-100* and *abi-2Δ1-157*) or an internal deletion (*abi-2Δ158-243*) of Abi-2
10 were subcloned in frame into pCGN vector and expressed as HA-tagged proteins in Cos cells by transient transfection. Cell lysates were prepared at 2½ days post-transfection and the lysates were incubated with GST alone (lanes 1, 4 and 7), GST-Abl
15 SH3 (lanes 2, 5 and 8), and GST-Abl (593-730) (lanes 3, 6 and 9). Bound proteins were separated on SDS-PAGE, transferred to nitrocellulose, and analyzed by Western blotting with the anti-HA monoclonal antibody.

20 Figure 7. Abi-2 is phosphorylated *in vitro* and *in vivo* by c-Abl

(A) *In vitro* phosphorylation: Immobilized GST alone (lanes 1, 3 and 5) or GST-Abi-2 (lanes 2, 4 and 6) was incubated at 4°C for 60 min. with *in vitro*
25 transcription/translation products (bottom panel) of c-Abl wild type (lanes 1 and 2), c-Abl K290R (lanes 3 and 4) and c-AblΔSH3Δ544-637 (lanes 5 and 6). Bound

proteins were subjected to *in vitro* phosphorylation at 30°C in the presence of [γ -³²P]ATP and MnCl₂. The phosphorylated proteins bound to beads were washed three times, separated on SDS-PAGE and visualized by autoradiography. (B) *In vivo* phosphorylation: Bosc 23 cells were transfected with pCGN vector alone (lane 1), pSR α /*c-abl* (lane 2), pCGN/*abi-2* (lane 3), and pSR α /*c-abl* plus pCGN/*abi-2* (lane 4). Cell lysates were separated on SDS-PAGE, transferred to nitrocellulose, and immunoblotted with the indicated antibodies. Immunoreactive proteins were visualized by ECL.

Figure 8. Expression of an *Abi-2* mutant deficient in binding to the *Abl* SH3 domain activates the tyrosine kinase and transforming properties of *c-Abl*

(A) Expression of *abi-2* Δ 1-157 in NIH3T3 cells. Cells were transfected with pCGN alone (lane 1) or pCGN/*abi-2* Δ 1-157 (lane 2). Following hygromycin selection, approximately 2x10⁵ cells were lysed in sample buffer and subjected to Western blot analysis using anti-HA antibody. The *Abi-2* Δ 1-157 protein is indicated with the arrow. (B) *Abi-2* Δ 1-157 reverses biologic selection against *c-Abl* overexpression. NIH3T3 cells expressing *Abi-2* Δ 1-157 or vector control were infected with retroviruses encoding the neo resistance gene alone or with *c-abl* wild type or *c-abl*K290R as indicated. After 2½ days, cells were

selected with G418. The left panels show anti-Abl Western blots of infected cells at 2½ days (top) or 22 days post-infection (bottom). c-Abl is indicated by the arrow. The right panel shows an anti-pTyr Western blot of the infected cells at 22 days post-infection. (C) Abi-2 Δ1-157 activates the c-Abl transforming activity. NIH3T3 cells were transfected with either pCGN vector (panels 1 to 3) or pCGN/abi-2Δ1-157 (panels 4 to 6). After selection with hygromycin, the cells were infected with retroviruses expressing neo (1 and 4), wild type c-Abl (2 and 6), c-Abl K290R (5), or v-Abl (3). At 2½ days post-infection the cells were incubated with media containing G418 and maintained under drug selection for 22 days. Morphological transformation was clearly observed in c-Abl infected NIH3T3 cells that had been transfected with Abi-2 Δ1-157 but not in cells expressing vector control (compare panels 2 and 6). (D) A model is proposed for the interaction of c-Abl and Abi-2 and the presence of c-Abl/Abi-2 complexes in unphosphorylated and tyrosine phosphorylated states. Activation of the c-Abl tyrosine kinase leading to tyrosine phosphorylation of Abi-2 may occur by a variety of events, some of which are listed in the box.

Figure 9. Effect of Abi-2 deletion mutants on BCR/Abl transforming activity.

(A) Diagram of the structural domains present in full length Abi-2 and truncated Abi-2 proteins (Abi-2 Δ 1-100 and Abi-2 Δ 1-157).

(B) The expression of Abi-2 deletion mutants in Cos cells is shown. Cos cells were transfected with vector alone (lane 1), pCGN/*abi-2* Δ 1-100 (lane 2) or pCGN/*abi-2* Δ 1-157 (lane 3) and subjected to Western blot analysis with anti-HA monoclonal antibody to detect the expression of HA-tagged Abi-2 mutants.

(C) Rat1 cells tranformed by p185 BCR/Abl were transfected with the indicated constructs. After five days of selection with hygromycin, the cells were plated in soft agar. The relative transforming activity was calculated by counting the number of colonies that grew in soft agar three weeks after plating.

Figure 10. Multiple Abi-related proteins may be expressed in mammalian cells.

Figure 11. Multiple alternatively spliced forms of Abi-2 may be expressed in mammalian cells. † = cloned by Wang et al (Oncogene 12:1921 (1996)).
HD = homeodomain homologous region; SH3 = Src homology 3 domain.

Figure 12. Chromosomal assignment of Abi-2 to human chromosome 2q33.

Figure 13. Correlation of human sequences detected by Abi-2 cDNA with human chromosomes in rodent X human somatic cell hybrids.

DETAILED DESCRIPTION OF THE INVENTION

5 The present invention relates to a novel Abl-interactor (Abi) protein and to a nucleic acid sequence encoding same. The Abi protein of the invention belongs to a family of SH3-containing, proline-rich proteins that interact with and/or
10 become phosphorylated by specific cytoplasmic tyrosine kinases. The present protein interacts with multiple domains of Abl and is a substrate for Abl tyrosine kinase activity. The discovery of the Abi protein of the invention provides new approaches to
15 deciphering the mechanism of Abl-induced carcinogenesis and to detecting, diagnosing and treating cancers, including leukemias, breast cancer, prostate cancer and colon cancer.

 The SH3 domain of the Abi protein of the
20 invention is at the C-terminus (see, for example, aa 346-397 of the Abi protein shown in Figure 1, A, designated Abi-2). Several proline-rich stretches are present (Figure 1, B) that constitute binding sites for SH3 domain-containing proteins and contain
25 the consensus PXXP sequence that is present in all high affinity SH3 ligands identified to date (Cohen

et al, Cell 80:237 (1995); Rickles et al, EMBO J. 13:5598 (1994)). A polyproline stretch is present upstream of the SH3 domain which could function as a transcriptional activation domain (Tanaka et al, Mol. Cell. Biol. 14:6046 (1994)). Sequences enriched in serine/threonine, glutamate/aspartate and proline residues, designated PEST regions, are also found in the present protein. PEST regions are identified in the central and C-terminal portions of the Abi protein (see Figure 1, B). The N-terminal region of the present protein is basic (eg, calculated pI about 11.4) and homologous to the DNA-binding sequence of homeodomain proteins (the protein of the invention is unique among the family to which it relates in having both an SH3 domain and a homeodomain homologous region). The C-terminal portion of the protein is acidic (eg, pI about 3.5). A serine-rich region is present in the central portion of the protein (see Figure 1, B). The Abi protein contains several (eg 9) serine/threonine residues followed by proline, indicative of phosphorylation by proline-directed protein kinases (Kemp and Pearson, Trends Biochem. Sci. 15:342 (1990)). Certain sites conform to the cdc 2 kinase consensus sequence Ser/Thr-Pro-X-basic (Moreno and Nurse, Cell 61:549 (1990)). There are also potential cAMP-dependent protein kinase sites (eg 11) and potential protein kinase C sites (eg 9) (Kemp and Pearson, Trends Biochem. Sci. 15:342

(1990)). Several tyrosines in the sequence are found in peptides that correspond to optimal peptide substrates for the Abl, Fps and Src protein tyrosine kinases (Songyang et al, Nature 373:536 (1995)).

5 A specific embodiment of the protein of the invention relates to a human Abi, for example, the protein designated Abi-2 having the amino acid sequence shown in Figure 1,A, or alternative spliced forms thereof (see splice site noted in Figure 2).

10 In addition to the Figure 1,A sequence, human Abi sequences include allelic variations, eg naturally occurring allelic variations, of the Figure 1,A sequence. The variant sequences retain the functional characteristics of the Figure 1,A sequence
15 and the structural characteristics shown in Figure 1,B. By way of example, it is noted that Figure 1,C illustrates variations that might be present in the N-terminal homeodomain-like homologous region of the present protein.

20 The present invention relates not only to the entirety of the Abi protein, for example, the Figure 1,A sequence or allelic variations thereof, but to portions thereof as well. The term "portions" relates to peptides and polypeptides of at least 10
25 or at least 16-18 amino acids in length, preferably, at least 30 or at at least 50 amino acids, more preferably at least 100 amino acids and most preferably at least 300 amino acids. Examples of

such portions include subsequences of the protein of the invention that comprise amino acid sequences corresponding to one or more of the domains depicted in Figure 1,B, particularly, the SH3 domain (eg about amino acids 360-397 of the Figure 1,A sequence), the tyrosine phosphorylation site (eg about amino acids 320-327 of the Figure 1,A sequence) or the homeodomain homologous region (eg about amino acids 55-117 of the Figure 1,A sequence), alone or in combination. The polypeptides Abi-2Δ1-100 and Abi-2Δ1-157 represent further examples of such portions.

In addition to the Abi protein, the present invention also relates to a nucleic acid sequence (DNA or RNA) encoding Abi, eg human Abi, and to fragments thereof suitable for use, for example, as probes or primers, of at least 15, preferably at least 30, more preferably at least 90, 150, 300, or 900 bases in length that encode the "portions" (eg domains) described above. In a specific embodiment, the invention relates to a nucleic acid sequences encoding the Figure 1,A amino acid sequence, alternative spliced forms thereof and portions thereof. In particular, the present invention relates to the Figure 2 nucleic acid sequence or fragments thereof (particularly that portion encoding Abi-2) or to a nucleic acid sequence substantially identical to the nucleic acid sequence of Figure 2. A "substantially identical" sequence is one the

complement of which hybridizes to the nucleic acid sequence of Figure 2 in 6 x saline/sodium citrate (SSC) containing 5 x Denhardt's reagent, 0.5% SDS, 100 µg/ml denatured salmon sperm DNA, and which
5 remains bound when subjected to washing at 68°C to 42°C with 2 x SSC containing 0.5% SDS. (For details of reagent preparation, etc, see Sambrook et al, Molecular Cloning, A Laboratory Manual, 2nd Edition). The invention also relates to nucleic acids
10 complementary to those described above.

The present invention also relates to a recombinant molecule (a construct) comprising a nucleic acid sequence as described above and to a host cell transformed therewith. Using methodologies
15 well known in the art, a recombinant molecule comprising a vector and a nucleic acid sequence encoding the Abi protein of the invention, or portion thereof as defined above, can be constructed. Vectors suitable for use in the present invention
20 include plasmid and viral vectors, for example, pGEX3X, pGEM, pAcGHLT, pCGN, pSRα, pSRαMSVtKneo and pJRQ. Appropriate vectors can be selected based on their compatibility with transformation into a selected host cell. The nucleotide sequence of the
25 invention can be present in the vector operably linked to regulatory elements, for example, a promoter. Suitable promoters include, but are not

limited to the SP6, T7, polyhedrin CMV, SR α , LTR and MMTV LTR promoters.

As indicated above, the recombinant molecule of the invention can be constructed so as to be suitable for transforming a host cell. Suitable host cells include prokaryotic cells, such as bacteria, lower eukaryotic cells, such yeast, and higher eukaryotic cells such as mammalian cells and insect cells. The recombinant molecule of the invention can be introduced into appropriate host cells using a variety of known methods.

The present invention further relates to a method of producing the Abi protein of the invention, or portions thereof as defined above. The method comprises culturing the above-described transformed host cells under conditions such that the encoding sequence is expressed and the protein thereby produced.

The Abi protein of the invention, or portions thereof as defined above, can be present in isolated form, for example, substantially free of proteins with which it is normally associated.

Advantageously, the protein is at least 90% pure, more preferably at least 95% pure (as determined by gel electrophoresis using an appropriate stain (eg coomassie blue or silver)). The proteins, polypeptides and peptides of the invention can be produced recombinantly using the nucleic acid

sequences as described above, or chemically using known methods. When prepared recombinantly, the protein of the invention can be produced alone or as a fusion product, for example, fused with a protein
5 such as glutathione-S-transferase (GST) or hemagglutinin (HA). For example, the coding sequence of the invention (eg the sequence encoding the human Abi) can be cloned in frame with a sequence encoding another protein (such as GST) and the fusion product
10 expressed in an appropriate host cell (see Figures 3, 4 and 5 and disclosure relating thereto).

The proteins, polypeptides and peptides of the invention can be used as antigens to generate Abi specific antibodies, particularly, antibodies
15 specific for human Abi (eg Abi-2). Methods of antibody generation are well known in the art. Both monoclonal and polyclonal antibodies are contemplated, as are antigen binding fragments thereof. Chimeric antibodies (eg humanized
20 antibodies) are also within the scope of the invention, appropriate splicing techniques being known in the art. One skilled in the art will appreciate that such antibodies (which can be labeled with a detectable label) can be used to selectively
25 identify and isolate the proteins of the invention, including mutant and/or phosphorylated (or unphosphorylated) forms of the Abi protein.

The results presented in the Examples that follow demonstrate that the protein of the invention, for example, Abi-2, forms a complex with c-Abl through direct contact with both the SH3 and C-terminal domains of c-Abl. Examination of the phosphorylation state of the endogenous c-Abl/Abi-2 complexes in unstimulated cells reveals that neither protein is tyrosine phosphorylated. Overexpression of c-Abl, however, leads to c-Abl activation and tyrosine phosphorylation of the co-expressed Abi-2 protein *in vivo*. While not to be viewed as limiting, a model is presented in Figure 8D for the existence of unphosphorylated and tyrosine-phosphorylated c-Abl/Abi-2 complexes.

The Abi protein (for example, Abi-2) may function to stabilize the inactive form of c-Abl or block access to critical cellular protein substrates of the c-Abl tyrosine kinase. A second role for the Abi protein of the invention is that of effector of the c-Abl protein tyrosine kinase. At least two tyrosines within Abi are found in sequences predicted to be optimal peptide substrates for the c-Abl tyrosine kinase (Songyang et al, Nature 373:536 (1995)). In the case of Abi-2, the optimal phosphorylation site corresponds to the tyrosine in the sequence YSDP just upstream of the SH3 domain (see Figure 1,A). These results indicate that the

Abl SH3 and C-terminal domains play a role in substrate recruitment.

In addition to the dual SH3-proline interactions within the c-Abl/Abi complex, contacts may be made between tyrosine phosphorylated sequences in Abi (eg Abi-2) and the Abl SH2 domain. It has been shown that protein tyrosine kinases preferentially phosphorylate peptide sequences that bind with high affinity to their own SH2 domains (Songyang et al, Nature 373:536 (1995)). This type of interaction may protect Abi-2 from dephosphorylation and strengthen the interaction between Abi-2 and c-Abl.

Expression of an Abi mutant protein that lacks the N-terminal homeodomain homologous region but retains the two domains implicated in binding to the Abl SH3 domain and Abl C-terminal sequences (eg Abi-2 Δ 1-100 (see Figure 9)), has been shown to inhibit the transforming activity of the Abl oncoprotein. It has also been shown that expression of an Abi mutant that lacks the sequences required for Abl SH3 domain binding while retaining binding to the C-terminal region of Abl (eg Abi-2 Δ 1-157 (see Figure 9)) does not inhibit Abl-transforming activity, in fact, exerts a stimulatory effect.

The effect of an Abi mutant protein on normal cAbl tyrosine kinase has also been examined (see Figure 8, Table 1). The Abi-2 Δ 1-157 mutant activates the tyrosine kinase and transformation activities of

cAbl in cells. The mutant protein may simply displace the endogenous Abi-2 protein and thereby allow constitutive phosphorylation of other protein targets by the c-Abl tyrosine kinase. Alternatively, binding of the mutant protein to the Abl C-terminus may activate its tyrosine kinase activity and transforming potential. These two possibilities may not be mutually exclusive. An additional mechanism whereby the mutant could elicit transformation is by retaining c-Abl in the cytoplasm and thereby keeping c-Abl outside of the nucleus. It has been proposed that the growth inhibitory activity of overexpressed c-Abl requires localization to the nuclear compartment (Sawyers et al, Cell 77:121 (1994)).

The identification of the Abi protein of the invention as a substrate of the c-Abl tyrosine kinase which, like c-Abl, is found in the cytoplasm and the nucleus, and the finding that alterations in Abi can activate the c-Abl transforming potential, provides new directions in the investigation of c-Abl functions and indicates a role for Abi in cancer. For example, mutations or deletions in Abi may be associated with the progression of Philadelphia-chromosome positive human leukemias from the chronic to the blast crisis phases of the disease. Alterations in Abi-2 may also be linked to the development of other leukemias and other forms of cancer. Abi proteins of the invention, and mutant

forms thereof including the truncated forms described above, can be used to design or identify therapeutic agents (eg mimetics) that stabilize Abl in a non-transforming form or specifically inhibit the transforming activity of oncogenic forms of the Abl tyrosine kinase.

It is noteworthy that the protein of the invention and HSI (Taniuchi et al, The EMBO J. 14:3664 (1995); Fukuda et al, Proc. Natl. Acad. Sci. USA 92:7302 (1995)) share certain structural similarities. Accordingly, functional similarities (eg role in B cell responsiveness and apoptosis) may also be shared. That being the case, it is contemplated that the protein of the invention, or appropriate portions thereof (eg those structurally similar to HSI), or mimetics thereof, can be used to modulate B cell responses. By way of example, it may be possible to transiently induce expression ex vivo to effect the conversion of stem cells to B cells.

The data provided in the Examples that follow demonstrate that alterations in the Abi protein of the invention can result in a deregulation of cAbl function as evidenced by results of transformation assays. Accordingly, the identification of mutations, deletions, amplifications or chromosomal rearrangements of the Abi protein of the invention can be used as an indicator of a neoplastic or preneoplastic condition. Such alterations can be

detected, for example, by comparing DNA, RNA and/or protein from tumor (or preneoplastic) tissue or cells with normal tissue or cells. Sequencing of the Abi gene from such tissue, when necessary or

5 advantageous, can be carried out, for example, using PCR or single-strand conformation polymorphism.

Protein sequencing can be carried out using known techniques. Further examination of the Abi protein in such tissue or cell samples can be used to

10 determine the state of tyrosine phosphorylation (eg using Western blotting with antiphosphotyrosine antibodies). An increase in Abi tyrosine phosphorylation may be indicative of deregulation of cAbl function. In this regard, cAbl protein can be

15 immunoprecipitated from lysates of tumors (or suspected preneoplastic tissue) and normal tissues and subjected to in vitro kinase assays with the Abi protein of the invention (eg GST-Abi-2) or portions thereof containing the tyrosine phosphorylation site

20 (in this regard, see description above of the tyrosine phosphorylation site of the Abi-2 protein and Kharbanda et al, Nature 376:785 (1995)).

Activation of the intrinsic cAbl tyrosine kinase results in enhanced phosphorylation of the substrate.

25 Figures 10 and 11 show the alternatively spliced forms of Abi-2 (an alternatively spliced form being designated Abi-2A). In Figure 11, four alternatively spliced forms of Abi-2 are shown and aligned.

Arg BP1A and Arg BP 1B were cloned and sequenced by Wang et al, Oncogene 12:1921 (1996). These molecules were identified by Wang et al in a screen for proteins that bound to the cAbl-related kinase, Arg.

5 Two cAbl binding sites, a N-terminal PXXP sequence and a C-terminal SH3 domain, exist in all four proteins. The N-terminal sequence of Abi-2 (dotted box) is specific because an insertion of an Abi-2 specific exon results in differential usage of the translation codon. The existence of the alternatively spliced forms of Abi-2 offers the possibility that the different forms serve purposes, eg that of an antagonist of cAbl kinase or that of an activator of cAbl kinase. That being the case, the expression of a specific form or forms may reflect a disease state, such as cancer.

The gene for human Abi-2 has now been mapped to human chromosome 2q33. Mutations in this region can serve as markers for disease, including cancer.

20 The Abi protein and the various alternatively spliced forms of Abi-2 of the invention may also exhibit an altered subcellular localization in preneoplastic or neoplastic tissue (such is known to be the case for p53 in breast cancer - see Moll et al, Proc. Natl. Acad. Sci. USA 89:7262 (1992)). Immunocytochemical analysis, for example, using affinity purified anti-Abi-2 antibodies, can be performed to determine whether the localization of

Abi protein in the suspected tissue is different from that of adjacent normal tissue.

As indicated above, the present invention includes within its scope methods of using the proteins, polypeptides and peptides of the invention (eg, recombinantly produced human Abi or portions (eg domains) thereof) to screen compounds for their ability to affect (eg inhibit) Abl oncogenic activity, for example, by stabilizing the cAbl inactive conformation or by blocking access of cAbl to its natural substrates. In one type of screening assay, compounds are tested for their ability to alter (eg inhibit) Abl transforming activity. For example, cells (eg NIH 3T3 cells) expressing both a cAbl encoding sequence and a sequence encoding a protein of the invention that converts cAbl into a transforming protein (eg an N-terminal truncated form of human Abi such as Abi-2Δ1-157) can be contacted with a compound the transformation-altering activity of which is to be tested. An inhibition of the transforming activity of cAbl (eg as determined by a reduction in the number of colonies having a transformed morphology) is indicative of a compound that is an inhibitor of cAbl-induced transformation and thus a compound potentially useful as a cancer chemotherapeutic agent. Based on this type of screen, compounds can be identified that mimic intact

Abi, for example, by restoring Abl to its inactive conformation and stabilizing it in that form.

The invention further relates to a screen for testing the ability of a compound to effect

5 transformation. In accordance with this screen, cells containing potentially oncogenic cAbl and an Abi protein of the invention or portion/mutant thereof that inhibits the oncogenic potential of the cAbl, are contacted with a compound the transforming
10 activity of which is to be tested. Transformation of the cells upon that contact is indicative of a potentially carcinogenic agent. Such agents may act by displacing the Abi protein of the invention from the Abi/cAbl complex.

15 Studies described in the Examples that follow demonstrate that expression of specific forms of the Abi protein of the invention can block the transforming activity of oncogenic Abl proteins.

Accordingly, the invention includes within its scope
20 a gene therapy approach to cancer treatment that involves introduction into cells, for example, tumor cells, of a nucleic acid sequence encoding the Abi protein of the invention, or portion/mutant thereof that blocks Abl transforming activity, under
25 conditions such that the protein is produced and the Abl transforming activity thereby blocked. Portions of the Abi protein suitable for use in such therapy can be determined in vitro using cultured cells that

represent a suitable model for the target tumor and transformation assays known in the art (eg growth in soft agar). Introduction of the nucleic acid into the target tissue can be effected using a delivery system selected based on the nature of the target tumor (for example, delivery can be effected using liposomes or expression vectors including viral vectors such as retroviruses, adeno- and adenoassociated viruses, herpes viruses, vaccine viruses and the like).

Also included within the scope of the invention are pharmaceutical compositions comprising the proteins, polypeptides or peptides, of the invention and compounds selected using the above-described screening protocols. In addition to the active agent, such compositions can include a pharmaceutically acceptable carrier, encapsulating agent (eg liposome), etc. The amount of active agent in the composition can vary with the agent, the patient and the effect sought. Likewise, the dosage regimen used will vary depending on the composition and the disease/disorder to be treated. Depending on the specific condition being treated and the formulation used, administration can be effected locally or systemically. Routes of administration include parenteral (eg intramuscular, subcutaneous, intravenous, intraperitoneal, intranasal or

intraocular), oral, rectal, transmucosal and intestinal.

The invention also includes within its scope kits suitable, for example, for use in carrying out the screening methodologies described above. Such kits can include nucleic acid sequences encoding the Abi protein of the invention, or portions thereof as described above, disposed within a container means. Alternatively, kits can include the peptide, polypeptide or protein of the invention. Such kits can be suitable for use in the analyses described above.

Certain aspects of the present invention are described in greater detail in the non-limiting Examples that follow.

EXAMPLES

The experimental protocols and materials described below are referenced in the specific Examples that follow.

Cells and Viruses

Recombinant c-Abl baculovirus was prepared as described (Pendergast et al, Cell 66:161 (1991)). Recombinant GAP baculovirus (Trahey et al, Science 242:1697 (1988)) was obtained from Dr. R. Clark and Dr. F. McCormick. Bosc 23 cells, obtained from

Drs. W.S. Pear and D. Baltimore (Pear et al, Proc. Natl. Acad. Sci. USA 90:8392 (1993)), were grown in DMEM with 10% fetal calf serum (FCS); COS cells were grown in DMEM plus 5% FCS; RAMOS cells were grown in RPMI 1640 plus 10% FCS; Sf9 insect cells were grown in Grace Media plus yeastolate and 10% FCS.

Antibodies

Polyclonal antibodies against Abi2 were prepared by immunization of New Zealand White female rabbits with a GST-Abi-2 fusion protein. Polyclonal antibodies and a mouse monoclonal anti-Abl antibody were previously described (Pendergast et al, Oncogene 4:759 (1989); Konopka et al, Cell 37:1035 (1984); Pendergast et al, Cell 66:161 (1991)). Antibodies against Ras GAP were obtained from Drs. R. Clark and F. McCormick. Monoclonal antibody against HA (12CA5) was purchased from Boehringer Mannheim. Monoclonal antibody against phosphorylated tyrosine (PY69) was obtained from ICN Biochemicals Inc.

Plasmid constructions

A cDNA fragment encoding the c-Abl SH3 domain (amino acids 47-147) was excised from pGEX3X(929-1029)P210[SH3] (Pendergast et al, Cell 66:161 (1991)) by Bam H1/Aat II restriction enzyme digestion and subcloned in frame into pPC62 (obtained from P.M. Chevray) (Chevray and Nathans, Proc. Natl. Acad.

Sci. USA 89:5789 (1992)). The resultant plasmid, designated pPC60/*ablSH3*, directs the synthesis of a fusion protein containing the Gal 4 DNA binding domain and the SH3 domain of c-Abl when expressed in yeast. The pPC60/*ablSH3* plasmid was used as the bait in the two-hybrid screening in yeast.

The pGEM vector (Promega) was used for *in vitro* transcription/translation of *abi-2*, *c-abl*, and their respective deletion mutants. The entire *abi-2* coding sequence was subcloned into the pGEM vector at the Bam HI site. This plasmid, designated pGEM/*abi-2*, was subsequently digested with Stu I to remove the sequence encoding amino acids 244-401 from *abi-2* and ligated to generate pGEM/*abi-2* Δ 244-401. To generate the SH3 domain deletion of Abi-2, the DNA sequence encoding amino acids 161-321 of Abi-2 was amplified by Polymerase Chain Reaction (PCR). The following forward and reverse oligonucleotide primers were used for PCR amplification: 5'-GTTGCAAGAAGAGAAAT-3' and 5'-GAAGATCTGGAGCCCACGGT-3'. A termination codon and restriction sites for Bam HI, Eco RI, and Hind III were engineered at the 3' of the PCR product to facilitate subcloning and to ensure appropriate termination of translation. The PCR-generated cDNA fragment was digested with Kpn I/Hind III and subcloned into pGEM/*abi-2* at the Kpn I and Hind III sites. The resultant plasmid was designated pGEM/*abi-2* Δ SH3.

To express *abi-2* in mammalian cells, a modified pCGN expression vector (Tanaka and Herr, Cell 60:375-386 (1990)) was used. The entire coding sequence of *abi-2* was subcloned in frame into pCGN at Bam H1 site downstream of the sequence encoding the HA tag. The resultant plasmid pCGN/*abi-2*, under the control of the CMV promoter, directs the synthesis of Abi-2 with an HA tag fused at its N-terminus.

Construction of pCGN/*abi-2* Δ 1-100 was performed by cutting the pGEM/*abi-2* plasmid with Xba 1, filling in with Klenow, and adding a Bam H1 linker to the 5' end of *abi-2*. The cDNA fragment with a 5' deletion was then subcloned in frame into the pCGN vector. To create pCGN/*abi-2* Δ 1-157, the pGEM/*abi-2* plasmid was cut with Kpn 1, blunted at the 5' end, and following addition of a Bam H1 linker at the 5', the Bam H1 cDNA fragment of *abi-2* was subcloned into pCGN. The plasmid pCGN/*abi-2* Δ 158-243 was created by an internal deletion of pGEM/*abi-2* at the Kpn 1 and StuI sites. The *abi-2* DNA fragment containing the internal deletion was then subcloned into pCGN.

pCGN/*c-abl* and pGEM/*abl* Δ SH3 were constructed as described previously (Pendergast et al, Proc. Natl. Acad. Sci. USA 88:5927 (1991)). To generate C-terminal deletion mutants of c-Abl, a PCR-directed mutagenesis strategy was employed as described (Dai et al., J. Biol. Chem. 267:19565 (1992)). The resultant plasmids, designated pGEM/*abl* Δ SH3 Δ 544-601

and pGEM/*abl*Δ*SH3*Δ544-637, respectively, bear double deletion of sequences encoding both SH3 and C-terminal amino acids as indicated.

The pGEX 3X vector (Pharmacia) was used to express a GST-Abi-2 fusion protein. The entire coding sequence of *abi-2* was subcloned in frame into pGEX 3X at the Bam HI site and the plasmid created was designated pGEX/*abi-2*. cDNA fragments encoding the SH3 and SH2 domains of c-Abl were subcloned in frame into pGEX 3X as described (Pendergast et al, Cell 66:161 (1991)). cDNA fragments encoding Abl C-terminal amino acids 593-1149, 593-730, and 731-1149 were excised from pGEM/*c-abl* by appropriate restriction enzyme digestions and subcloned in frame into pGEX 3X. These DNA fragments of *c-abl*, together with the full length *c-abl* and *c-abl*Δ*SH3*, were also subcloned in frame into pAS-CYH2 (obtained from S. Elledge) (Harper et al, Cell 75:805 (1993)) for testing the interaction by the yeast two-hybrid system.

Yeast two-hybrid screen

The yeast two-hybrid system was employed to screen a human lymphocyte cDNA library as described by Durfee et al, Genes Dev. 7:555 (1993)). The bait plasmid of cPC60/*abl*SH3 was cotransformed with the cDNA library into yeast strain Y190 (Harper et al, Cell 75:805 (1993)). 4 x 10⁵ transformants were screened for lac Z reporter gene expression by a

filter lift assay (Durfee et al, Genes Dev. 7:555 (1993)). The colonies that turned blue in 4 hours were replicated onto a minus tryptophan, leucine and histidine plate containing 25 mM-3-AT and grown at 30°C for 3 days. Total DNA was isolated from the colonies grown and used to transform the DH5 α bacterial strain by electroporation using a Bio-Rad GenePulser. Plasmid DNA was isolated from Ampicillin resistant colonies and tested for bait-dependent reporter gene expression by retransforming back into yeast strain Y190 with pAS-CYH plasmid alone or bait plasmid pPC60/*ab1SH3*. cDNAs that provided bait-dependent lac Z gene activation were subcloned into pBSK and subjected to dideoxy chain termination sequencing. Sequence analysis was performed with the Genetics Computer Group program (Pearson and Lipman, Proc. Natl. Acad. Sci. USA 85:2444 (1988)). FASTA searches for homologous sequences were performed against the SwissProt, PirProtein, and GenBank data libraries.

PCR amplification of *abi-2* 5' untranslated sequence was performed using the human lymphocyte cDNA library as template. The forward and reverse oligonucleotide primers used are: 5'-TGATGAAGATACCCACCA-3' and 5'-CTGGTCGTTCAAGGTTGGCTGGAGC-3'. Amplified cDNA fragments were purified by agarose gel electrophoresis and subcloned into pBSK. Plasmid DNA

from three independent colonies were prepared and sequenced. All three colonies contain cDNA inserts that represents the 5' untranslated sequence of *abl-2*.

5 *Subcellular fractionation*

Subcellular fractionation was performed as described (Dignam et al, Nucl. Acids Res. 11:1475 (1983)). Approximately 2×10^7 RAMOS cells were washed once with PBS and once with hypotonic buffer (10 mM
10 HEPES, pH7.9; 1.5 mM $MgCl_2$, 10 mM KCl, 0.2 mM PMSF, 0.5 mM DTT, 5 mg/ml leupeptin). Cells were swollen in a 3-fold packed cell volume of hypotonic buffer for 10 min, and homogenized by passing through a $27^{1/2}$ G needle 5-10 times. Release of cytosol was checked
15 under the phase microscope. Cell lysates centrifuged at 3300 g for 15 min to separate the nuclei from the cytosol/membrane fraction.

A second procedure, as described by Sakai et al, EMBO J. 13:3748 (1994) was also used with minor
20 modifications. Approximately 2×10^7 RAMOS cells were homogenized in 1 ml of hypotonic suspension buffer (20mM Tris, pH7.5 1 mM EDTA, 1 mM EGTA DTT, 1mM PMSF and 1mM Na_3VO_4) by passing through a $27^{1/2}$ G needle 5 times, followed by centrifugation at 1000 g for 15
25 min. The nuclear pellet and the supernatant containing cytosol and membrane fractions were suspended in equal volumes of 2XSB and subjected to

SDS-PAGE analysis. Isolation of chromatin was performed as described (Hancock et al, Methods Cell. Biol. 15:127 (1977)).

Expression and purification of GST-fusion proteins

5 GST fusion protein expression was induced as described (Pendergast et al, Proc. Natl. Acad. Sci. USA 88:5927 (1991)). The fusion proteins were purified on glutathione-Sepharose 4B (Pharmacia) beads as previously described (Pendergast et al, Cell
10 66:161 (1991)).

In vitro transcription and translation

In vitro transcription/translation was performed using the SP6 in vitro transcription/translation kit (Promega) according to manufacturer's specifications.
15 The reaction was incubated at 30°C for 2 hr and in vitro translated protein products were directly analyzed by SDS-PAGE or subjected to immunoprecipitation/binding assay prior to SDS-PAGE as indicated.

20 *Binding Assays*

Radiolabeled proteins from whole-cell lysates or translated in vitro in reticulocyte lysates were diluted 5-fold with incubation buffer (20 mM HEPES, pH7.0, 150 mM NaCl, 0.1% Triton X-100, 10% glycerol,
25 0.5 mM Na₃VO₄, 0.1 mM Na₂MoO₄, 20 mM NaF, 1 mM PMSF, 25

mg/ml leupeptin) and incubated with GST or GST fusion proteins attached to glutathione-Sepharose beads.

After incubation for 90 min at 4°C, the beads were washed four times with same buffer unless otherwise

5 indicated. The beads were boiled in 2 x sample buffer and bound proteins were analyzed by SDS-PAGE.

Immunoblotting

Proteins were separated on SDS-PAGE and transferred to nitrocellulose filters. Immunoblotting
10 was performed as described previously (Pendergast et al, Cell 75:175 (1993)). Immunoreactive proteins were visualized by the enhanced chemiluminescence (ECL) detection system (Amersham) according to manufacturer's specifications.

15 *In vitro kinase assay*

In vitro kinase assay was performed as described (Konopka et al, Cell 37:1035-1042 (1985)). Phosphorylation was carried out in the presence of [γ -³²P]ATP and MnCl₂ for 30 min at 30°C.

20 *Retrovirus Infections*

Helper-free retroviral stocks were prepared by transient hyperexpression in Bosc 23 cells as previously described (Muller et al, Mol. Cell. Bio. 11:1785 (1991); Pear et al, Proc. Natl. Acad. Sci. USA 90:8392 (1993)). Infection of NIH-3T3 fibroblasts
25

with the indicated retroviruses was performed using polybrene as previously described (Muller et al, Mol. Cell. Bio. 11:1785 (1991)). Following a 4 hr infection period, the cells were placed in fresh medium (DMEM + 10% FCS) and cultured for 2 1/2 days before selection with G418. Cells were selected for 22 days. Western blot analysis was performed on cell populations at 2 1/2 days post-infection and after 22 days of G418 selection. Cell transformation was quantitated by growth in soft agar as described previously (Muller et al, Mol. Cell. Biol. 11:1785 (1991)). The cells (1×10^4) were plated per 6-cm dish in duplicate. Agar colonies with a diameter of >0.5 mm were counted -2 weeks after plating the cells.

Example I

Cloning of an Abl Interacting Protein by the Yeast Two-Hybrid System

To identify proteins that interact with the Abl regulatory domains, the yeast two-hybrid system was employed (Fields and Song, Nature 340:245-246 (1989)). A human lymphocyte library was screened as genetic and biological data suggest that c-Abl plays a role in lymphocyte development (Schwartzberg et al, Cell 65:1165 (1991); Tybulewicz et al, Cell 65:1153

(1991); Caracciolo et al, Science 245:1107 (1989)).

Using the Abl SH3 domain as a bait, 24 positive clones were identified out of 4×10^5 transformants.

Among these, clone AS3B2 was demonstrated to confer a

5 bait plasmid-dependent expression of lac Z in yeast

strain Y190 and grew in his⁻, trp⁻, leu⁻ selection

plates with 25 mM 3-aminotriazole (3-AT). Nucleotide

sequence analysis revealed that AS3B2 contains a

single long open reading frame (ORF). The complete

10 coding sequence of AS3B2 encompasses 1203

nucleotides. The gene contained in AS3B2 was

designated *abi-2* for Abl Interacting Protein 1

(Aip-1) (Feller et al, Trends Biochem. Sci. 19:453-

458 (1994)). The amino acid sequence of Abi-2 is

15 shown in Figure 1,A and the nucleotide sequence of

abi-2 is shown in Figure 2.

Interestingly, the basic, N-terminal region of Abi-2 exhibits 40 to 50% similarity over a 53-amino acid stretch to the DNA-binding region of homeodomain

20 proteins (Figure 1, A, and C) (Rushlow et al, Genes

Dev. 1:1268 (1987); Scott et al, Biochem. Biophys.

Acta 989:25 (1989); Pabo and Sauer, Annu. Rev.

Biochem. 61:1053 (1992)). Homeodomain proteins have

been implicated in specifying positional information

25 in the embryo during development and in the control

of cell lineages by regulating the expression of cell

type-specific genes (Scott et al, Biochem. Biophys.

Acta 989:25 (1989)). It is significant that all of

the amino acids in the homeodomain that are implicated in contacting the DNA major groove are conserved in the basic region of Abi-2. Similarly, all of the amino acids that contact the DNA backbone with the exception of an invariant tryptophan, are present in this domain of Abi-2 (Figure 1C) (Pabo and Sauer, Annu. Rev. Biochem. 61:1053 (1992)).

Search of the database for homologous sequences revealed that the *abi-2* DNA sequence is 70% identical to that of the *Xenopus laevis xlan 4* DNA (Redy et al, Mech. Dev. 39:143 (1992)). The predicted amino acid sequence of the *Xlan 4* protein is 93% identical to that of the last 286 amino acids of Abi-2.

Remarkable conservation of the SH3 domain, PEST sequences, serine-rich region, proline-rich stretches and phosphorylation sites is observed among the human Abi-2 and predicted *Xenopus Xlan 4* proteins. The *xlan 4* gene is expressed as a maternal transcript and localizes in the animal pole region of the oocyte.

The expression of *xlan 4* is developmentally regulated (Redy et al, Mech. Dev. 39:143 (1992)). More recently, a mouse gene with high homology to the human *abi-2* was cloned as cDNA encoding an Abl-binding protein. The corresponding protein was designated Abl interactor 1 (Abi-1) and its predicted amino acid sequence is 65% identical to that of Abi-2.

Example II

Expression of Abi-2 in Human Tissues

To investigate the expression pattern of *abi-2*, Northern blot analysis was performed on poly A
5 selected RNA from multiple human tissues. Using the *abi-2* cDNA as a probe, two transcripts, sized at 7Kb and 1.9 Kb respectively, were detected in all tissues examined (Figure 1D). The abundance of the two transcripts varies among tissues. The expression of
10 the 7 Kb transcript appears constant in most tissues examined with the exception of colon and peripheral blood leukocytes where low levels of the transcript are detected. Although detectable in almost all tissues examined, 1.9 Kb transcript is relatively
15 abundant in testes, ovary, thymus, and colon with lower but detectable levels in prostate, peripheral blood leukocytes and spleen. It is most likely that *abi-2* corresponds to the 1.9 kb transcript. The 7 Kb transcript may be the product of a related gene or an
20 alternative spliced form of the *abi-2* gene.

Example III

Expression of the Abi-2 Protein

The complete coding sequence of *abi-2* was subcloned into the pGEM vector downstream of the SP6 promoter and the resulting construct was then used for *in vitro* transcription/translation. Analysis of the *in vitro* transcription/translation products by SDS-PAGE revealed the presence of three major protein bands with apparent molecular weights of 75-, 55-, and 40-KDa (Figure 3A, lane 3). All three proteins were recognized by antibodies to Abi-2 (Figure 3a, lane 5) but not by the corresponding pre-immune serum (Figure 3A, lane 4). The two lower sized proteins (55- and 40-KDa) may represent products from an internal initiation site or proteolytic degradation fragments. Several potential translational initiation sites are found in the *abi-2* nucleotide sequence. Using the first ATG as the initiation codon, the calculated molecular weight of Abi-2 is 44-Kda. Aberrant protein migration has been reported for several proteins which, like Abi-2, are proline-rich and contain stretches of basic/acidic amino acids (Matthews et al, Mol. Cell. Biol. 12:2396 (1992)).

The expression of Abi-2 was next investigated in human Bosc 23 cells (Pear et al, Proc. Natl. Acad.

Sci. USA 90:8392 (1993)) following transfection of
abi-2 cloned in a mammalian expression vector under
the control of the CMV promoter (Tanaka and Herr,
60:375 (1990)). A sequence coding for the influenza
virus hemagglutinin epitope (HA) was fused in frame
at the 5' end of the abi-2 sequence for detection of
the gene product with monoclonal antibody (12CA5)
against the HA-tag. The construct directs production
of two broad protein bands of approximately 75- and
55-KDa in the Bosc 23 human embryonic kidney cells.
The two proteins were recognized in Western blot
analysis with monoclonal antibody to the HA epitope
tag (Figure 3B, lane 2) as well as polyclonal
antibodies to Abi-2 (Figure 3B, lane 3). Expression
of endogenous Abi-2 protein was investigated using
the human B lymphoid cell line, RAMOS. Western blot
analysis with antibodies to Abi-2 revealed two
proteins with apparent molecular weights of 75- and
55-KDa (Figure 3B, lane 4). The two proteins
co-migrated with the proteins expressed in Bosc 23
cells following transfection with the abi-2 cDNA
(Figure 3B, lane 3).

Example IV

Subcellular Localization of Abi-2

The subcellular localization of Abi-2 was determined by cellular fractionation employing two distinct procedures (Dignam et al, 11:1475 (1983); Sakai et al, EMBO J. 13:3748 (1994)). Endogenous Abi-2 protein from RAMOS cells was shown to be localized primarily in the nucleus, with lower but significant levels in the cytosol and membrane fractions (Figure 3C). Interestingly, the nuclear Abi-2 protein was mostly full length (75 KDa), while equal amounts of the 75 KDa and 55 KDa forms of Abi-2 were present in the cytoplasm (Figure 3C, lanes 2 and 3). These results suggest that the nuclear pool of Abi-2 may not be accessible to the activity of specific proteolytic enzymes present in the cytoplasm or alternatively, that Abi-2 is protected from degradation in the nucleus by formation of specific protein complexes. The nuclear Abi-2 protein appears to be associated with chromatin. Significantly, the subcellular distribution of Abi-2 is similar to that of c-Abl (Van Etten et al, Cell 58:669 (1989); Van Etten et al, J. Cell. Biol. 124:325 (1994); Wetzler et al, J. Clin. Invest. 92:1925 (1993)).

Example V

Abi-2 binds to c-Abl In Vitro and In Vivo

It has been shown that Abi-2 binds to the Abl SH3 domain in yeast. To determine whether Abi-2 can interact with the full length c-Abl protein, a GST-Abi-2 fusion protein was used in an in vitro binding assay with full length c-Abl produced in baculovirus-infected insect cells (Pendergast et al, Cell 75:175 (1993)). As shown in Figure 4A, the GST-Abi-2 protein interacts with full length c-Abl in solution. The binding of Abi-2 to c-Abl appears to be selective as shown by the failure of Abi-2 to form a complex with another SH3-containing protein, the guanosine triphosphatase-activating protein (GAP) of Ras (Figure 4A, lanes 4-6) (Trahey et al, Science 242:1697 (1988)).

To examine the interaction of Abi-2 with c-Abl in vivo antibodies were developed to the Abi-2 protein. Interestingly, analysis of the Abi-2 protein by SDS-PAGE revealed that Abi-2 migrates aberrantly. While the calculated molecular weight of the protein encoded by the abi-2 cDNA is 44 kDa, the Abi-2 protein produced in bacteria or translated in vitro in a reticulocyte lysate migrates with an apparent molecular weight of 55 kDa (Figure 6B). In addition to the major 55 kDa band, two proteins of

44 kDa and 75 kDa are obtained following *in vitro* transcription/translation using the *abi-2* cDNA which are recognized by an anti-Abi-2 polyclonal antibody raised against full length Abi-2. The smaller 44 kDa protein is likely to be produced by translation from an internal initiation codon because it cannot be recognized by antibodies specific to the Abi-2 N terminus. The 75 kDa protein may result from additional post translational modifications. A 75 kDa protein is the predominant product obtained following transfection of the *abi-2* cDNA in Bosc23 human embryonic kidney cell (Figure 4B and C). The 75 kDa protein is recognized by anti-Abi-2 antibodies. The 75 kDa protein and a minor 55 kDa protein are also detected with the anti-Abi-2 polyclonal antibodies in lysates from human B lymphoid cells.

Interaction of Abi-2 with c-Abl *in vivo* was examined following overexpression of wild type c-Abl and Abi-2 in human Bosc 23 cells and immunoprecipitation with antisera to Abi-2 or c-Abl. Bosc 23 cells were transfected with pCGN/*abi-2* and pSR α /*c-abl* mammalian expression plasmids. After 2½ days, the cells were lysed and the lysates were incubated with anti-Abl, anti-Abi-2 or pre-immune sera. The immunoprecipitates were then subjected to *in vitro* kinase assays with [γ^{32} -P] ATP to radiolabel the proteins. A 145 kDa protein was precipitated by

the anti-Abi-2 antibody but not the corresponding pre-immune sera (Figure 4B, lanes 1 and 2). This 145 kDA protein comigrated with a protein of the same size which was immunoprecipitated with anti-Abl antibodies from the same cells (Figure 4B, lane 3). The identity of the 145 kDA protein as c-Abl was confirmed by subjecting the precipitated proteins to a second round of immunoprecipitation with anti-Abl antibodies. A 145 kDA protein immunoreactive with anti-Abl antibodies is observed following incubation of a portion of the samples corresponding to lanes 2 and 3 in Figure 4B with anti-Abl antibodies (Figure 4C). Thus, the c-Abl wild type protein is precipitated with antibodies to Abi-2 as detected by its *in vitro* autophosphorylation activity.

A radiolabeled protein of approximately 75 kDA was detected in both anti-Abi-2 and anti-Abl immunoprecipitates from lysates of Bosc 23 cells co-transfected with the pCGN/*abi-2* and pSR α /*c-abl* expression plasmids (Figure 4B, lanes 2 and 3). To examine whether this protein was Abi-2, a portion of the immunoprecipitates was boiled in the presence of SDS to denature the proteins and disrupt protein/protein interactions, diluted with buffer lacking SDS and then incubated with anti-Abi-2 antibodies or the corresponding pre-immune sera. A 75 kDA protein was precipitated with anti-Abi-2 but not the pre-immune sera from both, the anti-Abi-2 and

anti-Abl immunoprecipitates of the Bosc 23 cell lysates (Figure 4C, lanes 1, 2, 4 and 5). These results show that full length Abi-2 interacts with c-Abl following overexpression of both proteins in Bosc 23 cells and that Abi-2 becomes phosphorylated in an *in vitro* kinase assay in the presence of the c-Abl tyrosine kinase.

It has also been observed that endogenous Abi-2 co-immunoprecipitates with the endogenous c-Abl protein in B cell lysates. However, the reciprocal co-precipitation of c-Abl with anti-Abi-2 antibodies has not been shown in lysates from these cells. The inability of the anti-Abi-2 antibodies to co-precipitate endogenous c-Abl in B cells may result from association of the Abi-2/c-Abl complex with other proteins in the B cell lysates that mask the antigenic epitopes recognized by the anti-Abi-2 antibodies. Alternatively, the phosphorylation state of the c-Abl and Abi-2 proteins in the complex may result in differential recognition of the complexed Abi-2 protein by the corresponding antibodies. It has been shown that, unlike endogenous c-Abl, the overexpressed c-Abl protein is tyrosine phosphorylated and activated (Pendergast et al, Proc. Natl. Acad. Sci. USA 88:5927 (1991)). The overexpressed and activated c-Abl kinase may phosphorylate Abi-2 in the Bosc 23 cells allowing the

appropriate antigenic sequences to become accessible to the anti-Abi-2 antibody.

Example VI

Abi-2 Binds to Multiple Surfaces on the c-Abl Protein

5 To confirm that the Abl SH3 domain was responsible for the interaction between c-Abl and Abi-2, a mutant c-Abl protein lacking the SH3 domain was examined to determine whether it was deficient in binding to Abi-2. Surprisingly, the c-Abl Δ SH3
10 mutant bound to Abi-2 as efficiently as wild type c-Abl (Figure 5A). This result suggested that, in addition to the SH3 domain, other sequences in c-Abl may participate in binding to Abi-2. Using both the yeast two-hybrid system and *in vitro* binding assays,
15 a second region in the C-terminus of c-Abl was identified that interacted strongly with Abi-2 (Figure 5B and Figure 5D, lanes 9 and 10). The Abi-2-binding region in the C-terminus of c-Abl maps to sequences near the nuclear localization signal.
20 Recently, it was reported that this region of c-Abl can bind to the SH3-containing Crk, Grb2 and Nck adaptor proteins (Ren et al, Genes Dev. 8:783 (1994)). Three distinct proline-rich binding sites for the adaptor proteins were found within this
25 region of c-Abl. The presence of an SH3 domain in

Abi-2 suggested that Abi-2 may also interact with c-Abl by direct binding of the Abi-2 SH3 domain with the proline-rich sequences in the C-terminus of c-Abl.

5 To more precisely define the Abi-2 binding site in the Abl C-terminus, additional c-Abl mutants were created and tested for binding to Abi-2 (Figure 5C). Two of the three proline rich stretches identified in c-Abl that bind to SH3 domains are found upstream of
10 the nuclear localization sequence, while the third proline stretch is downstream of this sequence (Ren et al, Genes Dev. 8:783 (1994)). Deletion of the two proline-rich sites upstream of the nuclear
localization signal (Δ 544-601) did not diminish
15 binding of c-Abl Δ SH3 to Abi-2 (Figure 5C, lane 8). In contrast, deletion of additional sequences including the third proline-rich site (Δ 544-637), completely abolished binding of c-Abl Δ SH3 to Abi-2 (Figure 5C, lane 11). These results indicate that
20 Abi-2 binds to c-Abl by interacting directly with the Abl SH3 domain and a proline-rich stretch downstream of the nuclear localization signal in the Abl C-terminus.

Example VII

Specificity of Binding of Abi-2 to the Abl SH3 Domain

Specificity in the interaction of the various SH3 domains with their proline-rich targets is necessary for the proper transmission of intracellular signals involving distinct signal transduction pathways and protein regulatory cascades (Pawson, Nature 373:573 (1995); Cohen et al, Cell 80:237 (1995)). To determine whether the interaction between the Abl SH3 domain and Abi-2 is selective, a number of SH3 domains from distinct proteins were compared for their ability to bind to Abi-2. No interactions were detected between Abi-2 and the SH3 domains of GAP, Crk (N-terminal), Crk (N-terminal and C-terminal), Grb2 (N-terminal), and the p67 protein from neutrophils (Figure 5D, lanes 4 to 8). Also, no interaction was detected between Abi-2 and the SH3 domain of the Src protein tyrosine kinase. These results indicate that the interaction between Abi-2 and the Abl SH3 domain is highly specific and that Abi-2 contacts a second region in the C-terminus of c-Abl with high affinity.

Example VIII

*Binding of c-Abl to the Proline-Rich N-Terminus
and C-Terminal SH3 Domain of Abi-2*

To map the sites on Abi-2 that interact with
5 c-Abl, a series of Abi-2 deletion mutants was
generated (Figure 6A) and tested for binding to GST-
fusion proteins containing the Abl SH3 domain and Abl
C-terminal sequences (Figures 6B and 6C). Wild type
(full length) and C-terminal truncated Abi-2 proteins
10 were synthesized by *in vitro*
transcription/translation in a rabbit reticulocyte
lysate. The predominant *in vitro*
transcription/translation products generated from the
full length *abi-2* cDNA migrate with apparent
15 molecular weights of 55 and 44 kDA (Figure 6B).
Deletion of the Abi-2 SH3 domain abrogates binding to
the proline-rich Abl C-terminal sequences
encompassing amino acids 593 to 730 (Figure 6B,
lane 6). Further deletion of sequences upstream of
20 the Abi-2 SH3 domain that contain a polyproline
stretch and a PEST domain does not affect binding to
the Abl SH3 domain (Figure 6B, lane 8).

To identify the proline-rich sequences in Abi-2
that mediate its binding to the Abl SH3 domain, Abi-2
25 mutant proteins were generated containing deletions
of proline-rich sequences localized in the N-terminal

or central regions of the protein (Figures 6A and 6C). The Abi-2 proteins were produced in mammalian COS cells and tested for binding to Abl sequences. As shown in Figure 6C, deletion of the most
5 N-terminal proline-rich stretch of Abi-2 eliminates binding to the Abl SH3 domain (Figure 6C, lane 5). This 10-amino acid proline-rich stretch exhibits strong similarity to the Abl SH3-binding site in 3BP-1 (Cohen et al, Cell 80:237 (1995)). Thus, the
10 results show that the c-Abl tyrosine kinase contacts Abi-2 at two sites: an N-terminal proline-rich stretch and the Abi-2 SH3 domain. A model for the interaction between c-Abl and Abi-2 is shown in Figure 8D.

15

Example IX

Abi-2 is a Substrate for the c-Abl Tyrosine Kinase In Vitro and In Vivo

The strong association between Abi-2 and c-Abl suggested that Abi-2 may be a target for tyrosine
20 kinase activity of c-Abl. Additional support for this suggestion was provided by the observation that Abi-2 became phosphorylated in immune complex *in vitro* kinase assays following co-precipitation with the c-Abl tyrosine kinase from Bosc 23 cell lysates co-
25 expressing the two proteins (Figure 4B and C). To

determine whether c-Abl can phosphorylate purified Abi-2 protein, wild type and kinase defective forms of c-Abl were synthesized by *in vitro* transcription/translation in a reticulocyte lysate (Figure 7A) and tested for their ability to phosphorylate purified GST or GST-Abi-2 proteins *in vitro*. Abi-2 was phosphorylated to high levels by wild type but not kinase defective c-Abl (Figure 7A). A c-Abl mutant protein, c-Abl Δ SH3 Δ 544-637 that retains tyrosine kinase activity but is defective in binding to Abi-2 (Figure 5C), did not phosphorylate Abi-2 in this assay (Figure 7A, lane 6).

It was then determined whether Abi-2 could be phosphorylated *in vivo* by c-Abl following expression in Bosc 23 cells. The cells were cotransfected with pCGN/*abi-2* and pSR α /*c-abl* plasmids. Two days post-transfection, the cells were lysed and the lysates subjected to Western blot analysis with anti-phosphotyrosine antibodies. Western blotting with anti-Abl and anti-HA antibodies was also performed to detect the expression of c-Abl and the HA-tagged Abi-2, respectively (Figure 7B). Coexpression of Abi-2 with wild type c-Abl resulted in a marked increase in Abi-2 tyrosine phosphorylation (Figure 7B, lane 4). These data show that Abi-2 can be phosphorylated by the activated c-Abl tyrosine kinase *in vivo*.

Example X

*Expression of an Abi-2 Mutant Protein Uncovers
the Transforming Activity of c-Abl*

The findings that Abi-2 binds to c-Abl via dual
5 SH3 domain/proline-rich sequence interactions and
that Abi-2 is a substrate of the c-Abl tyrosine
kinase strongly suggested that Abi-2 modulates the
biological properties of c-Abl in the cell. Two
biological assays are available to examine the
10 effects of Abl expression in vivo. First,
overexpression of c-Abl wild type in NIH-3T3 cells
has been shown to elicit growth arrest and result in
counterselection of the expression of the c-Abl
protein (Jackson et al, EMBO J. 12:2809 (1993);
15 Sawyers et al, Cell 77:121 (1994)). Second, specific
alterations in the c-Abl structure have been shown to
result in cell transformation (Wang, Curr. Opin.
Genet. Dev. 3:35 (1993). To examine whether Abi-2
can affect the biological properties of c-Abl,
20 NIH-3T3 cells were transfected with hygromycin-
containing mammalian expression vectors encoding full
length or truncated forms of Abi-2. Expression of
full length Abi-2 could not be detected following
transfection and hygromycin drug selection. Like the
25 Abl proteins, full length Abi-2 appears to exert
cytotoxic, and possibly cytostatic, effects on cells

following overexpression (Ziegler et al, Cell 27:477 (1981); Renshaw et al, EMBO J. 11:3941 (1992); Jackson et al, EMBO J. 12:2809 (1993); Sawyers et al, Cell 77:121 (1994)). In contrast, an N-terminal truncated form of Abi-2 lacking the homeodomain homologous region and the proline-rich sequences required for interaction with the Abl SH3 domain (Abi-2 Δ 1-157) was readily expressed in NIH-3T3 cells (Figure 8A). Cells expressing the Abi-2 Δ 1-157 mutant and cells expressing the vector alone were infected with retroviruses encoding wild type c-Abl or a kinase-defective form of c-Abl, c-Abl K290R (Figure 8B). A fraction of the cells was collected at 2½ days post-infection, lysed and subjected to western blot analysis with anti-Abl antibodies. Similar levels of c-Abl wild type and c-Abl K290R proteins were detected in control NIH-3T3 cells and in NIH-3T3 that stably expressed the Abi-2 Δ 1-157 mutant (Figure 8B, upper left panel). Surprisingly, analysis of drug-selected cells at 22 days post-infection revealed that while the c-Abl wild type protein was counter selected in control NIH-3T3 cells in accordance with previous observations (Sawyers et al, Cell 77:121 (1994)), c-Abl was expressed to high levels in NIH-3T3 cells that co-expressed the Abi-2 Δ 1-157 mutant protein (Figure 8B, lower left panel). As shown previously, the kinase-defective c-Abl K290R protein was not counter selected in NIH-3T3 cells,

and similar levels of this protein were detected in control and Abi-2 Δ 1-157-expressing cells following drug selection at 22 days post-infection (Figure 8B, lower left panel). To determine whether co-

5 expression of the truncated form of Abi-2 and c-Abl wild type resulted in activation of the tyrosine kinase activity of c-Abl, the lysates were subjected to western blot analysis with antiphosphotyrosine antibodies. As shown in Figure 8B, right panel,
10 expression of Abi-2 Δ 1-157 activates the tyrosine kinase activity of c-Abl in NIH-3T3 cells. No enhanced tyrosine phosphorylation is detected in cells co-expressing Abi-2 Δ 1-157 and a kinase defective form of c-Abl (Figure 8B, right panel).

15 A striking difference was observed among the cells co-expressing c-Abl wild type and Abi-2 Δ 1-157 and those cells expressing Abi-2 Δ 1-157 alone, c-Abl alone, vector control, or co-expressing Abi-2 Δ 1-157 and a kinase-defective form of c-Abl (Figure 8C).

20 Cells that co-expressed Abi-2 Δ 1-157 and c-Abl wild type were morphologically transformed and exhibited a similar morphology to those NIH-3T3 cells expressing the v-abl oncoprotein (Figure 8C, panels 3 and 6).

To directly examine whether the cells co-expressing
25 Abi-2 Δ 1-157 and cAbl wild type were transformed, colony formation in soft agar was assayed as a measure of anchorage-independent growth (Muller et al, Mol. Cell. Biol. 11:1785 (1991)). As shown in

Table 1, cells co-expressing the Abi-2 Δ 1-157 mutant and cAbl wild type exhibit a dramatic increase in soft agar plating efficiency compared to cells expressing either protein alone or to cells
5 expressing both the Abi-2 Δ 1-157 mutant and a kinase defective form of cAbl. These results demonstrate that expression of the Abi-2 Δ 1-157 mutant protein activates the tyrosine kinase and transforming activities of c-Abl in NIH-3T3 cells.

TABLE 1

Transformation of NIH 3T3 cells by co-expression
of c-Abl and a truncated Abi-2 protein

Retroviruses ^a	No. of Agar Colonies ^c	
	NIH 3T3 vector ^b	NIH 3T3/Abi-2Δ1-157 ^b
c-Abl wild type	3	563
c-Abl K290R	2	3
Tkneo	4	3

^aHelper-free retroviruses were prepared by transient overexpression in Bosc 23 cells.

^bNIH 3T3 cells stably transfected with either pCGN vector alone or pCGN/abi-2Δ1-157 were infected with the indicated helper-free retroviruses. At 2½ days post-infection the cells were selected with G418 and cultured for three weeks in the presence of the drug.

^cNumber of agar colonies per 10⁴ cells. The average frequency of colony formation in soft agar was determined from two plates per assay. Colonies with a diameter of >0.5mm were counted approximately two weeks after plating the cells.

Example XI

Effect of Abi-2 Deletion Mutants on
BCR/Abl Transforming Activity

To examine the effects of expressing various
5 Abi-2 forms on the transforming activity of BCR-Abl,
Rat1 cell that stably expressed P185 BCR-Abl (Muller
et al, Mol. Cell. Biol. 11:1785 (1991)) were
employed. These cells are transformed as indicated
by their ability to grow in soft agar. The Abi-2
10 proteins encoding sequences cloned in the pCGN
expression vector were introduced. As shown in
Figure 9(A) and (B), the truncated Abi-2 proteins
lacking N-terminal sequences were expressed to high
levels following transient transfection in COS cells.
15 Transfection of the truncated Abi-2 proteins in
Rat1/P185 BCR-Abl cells, followed by drug
(Hygromycin) selection, resulted in the stable
expression of the truncated $\Delta 1-100$ and $\Delta 1-157$ Abi-2
proteins. In contrast, no clones expressing the full
20 length Abi-2 protein were generated. The Rat1/BCR-
Abl cells expressing the indicated proteins were then
plated in soft agar. Agar colonies that grew after
3 weeks were counted.

As shown in Figure 9(C) expression of the Abi-2
25 mutant protein, Abi-2 $\Delta 1-100$, which lacks the
N-terminal homeodomain homologous region but retains

the two domains implicated in binding to the Abl SH3 domain and Abl C-terminal sequences, inhibits the transforming activity of the BCR/Abl oncoprotein by 70% in Rat1 cells. Interestingly, expression of the

5 Abi-2 mutant, Abi-2 Δ 1-157, which lacks the sequences required for Abl SH3 domain binding while retaining binding to the C-terminal region of Abl, does not inhibit BCR/Abl-transforming activity and exerts a 2- to 3-fold stimulatory effect.

10 Example XII

Chromosomal Assignment of Abi-2

A mapping panel (No. 1) consisting of DNA from seventeen mouse-human and one Chinese hamster-human hybrids was obtained from NIGMS repository (Camden, NJ). Characterization and human chromosome content

15 are described in detail in the NIGMS catalog. DNA samples were digested with Bgl II, separated by electrophoresis on agarose gels, transferred to Hybond nylon filter (Amersham) and hybridized to the

20 32 P-labeled cDNA probe as described (Yang-Feng et al, Am. J. Hum. Genet. 37:1117 (1985)). The cDNA identified seven human genomic fragments, 25, 12.5, 6.8, 6.1, 5.1, 4.2 and 3.3 kb, and detected six fragments, 16.5, 10.5, 7.6, 4.3, 3.9 and 3.2 kb, in

25 the mouse DNA and five fragments, 6.6, 3.5, 3.2, 2.9,

and 0.4 kb, in the hamster DNA. The 3.3 kb human fragment could not be clearly distinguished from the mouse or hamster 3.2 kb band, therefore, could not be scored for in hybrids. The results of discordancy analysis are summarized in Figure 13. The Abi-2 sequence showed perfect segregation with human chromosome 2.

Regional mapping of Abi-2 was accomplished by in situ hybridization. The cDNA clone was nick-translated with $\{^3\text{H}\}$ dCTP and $\{^3\text{H}\}$ TTP to a specific activity of 2.5×10^7 cpm/ μg and hybridized to human metaphase chromosomes at a concentration of 25ng/ml. Of 138 grams over 50 cells analyzed, 20 (14.5%) were found to be at chromosome 2q33 and no other site was labeled above the background.

* * *

All documents cited above are hereby incorporated in their entirety by reference.

One skilled in the art will appreciate from a reading of this disclosure that various changes in form and detail can be made without departing from the true scope of the invention.

WHAT IS CLAIMED IS:

1. An isolated nucleic acid encoding mammalian Abi protein.

2. The nucleic acid according to claim 1 wherein said Abi protein is human Abi protein.

3. The nucleic acid according to claim 2 wherein said human Abi protein has the amino acid sequence set forth in Figure 1,A.

4. An isolated nucleic acid encoding mammalian Abi protein, or portion thereof of at least 90 consecutive bases, or complement thereof.

5. The nucleic acid according to claim 4 wherein said mammalian Abi protein is human Abi protein.

6. The isolated nucleic acid according to claim 4 wherein the nucleic acid encodes the amino acid sequence set forth in Figure 1,A, or portion thereof of at least 16 amino acids.

7. The isolated nucleic acid according to claim 6 wherein the nucleic acid has the sequence shown in Figure 2, or a sequence substantially

identical thereto, or portion thereof of at least 90 consecutive bases.

8. The isolated nucleic acid according to claim 7 wherein said nucleic acid has the sequence shown in Figure 2, or portion thereof of at least 90 consecutive bases.

9. The isolated nucleic acid according to claim 8 wherein the nucleic acid has the sequence shown in Figure 2.

10. A recombinant molecule comprising said nucleic acid according to claim 4 and a vector.

11. The recombinant molecule according to claim 10 further comprising a promoter operably linked to said nucleic acid sequence.

12. A host cell comprising said recombinant molecule according to claim 10.

13. A method of producing mammalian Abi protein, or portion thereof, comprising culturing said host cell according to claim 12 under conditions such that said nucleic acid is expressed and said mammalian Abi protein, or portion thereof, is thereby produced.

14. An Abi protein produced by the method according to claim 13.

15. An isolated Abi protein, or portion thereof, that modulates cAbl transforming activity.

16. The protein according to claim 15 wherein said protein is a mammalian protein.

17. The protein according to claim 15 wherein said protein has the sequence given in Figure 1.

18. A cell comprising nucleic acid sequences encoding normal cAbl and a mutant Abi protein that renders said cAbl oncogenic.

19. A cell comprising nucleic acid sequences encoding an oncogenic form of cAbl and a mutant Abi protein that inhibits the oncogenic activity of said form of cAbl.

20. A cell comprising an oncogenic form of cAbl and a sufficient amount of normal Abi protein or mutant thereof to inhibit the oncogenic activity of said form of cAbl.

21. The cell according to claim 20 wherein said Abi protein is normal Abi protein.

22. A method of screening a compound for the ability to modulate Abl oncogenic activity comprising:

i) contacting an inactive cAbl molecule with a mutant Abi protein that effects the conversion of said inactive cAbl to oncogenic cAbl upon complexing therewith,

ii) contacting a complex resulting from step (i) with a compound the modulating activity of which is to be tested, and

iii) determining whether contact with said compound converts said oncogenic cAbl to inactive cAbl.

23. The method according to claim 22 wherein said complex is present in a cell.

24. A kit comprising the nucleic acid according to claim 4 disposed within a container means.

25. A kit comprising the protein according to claim 15 disposed within a container means.

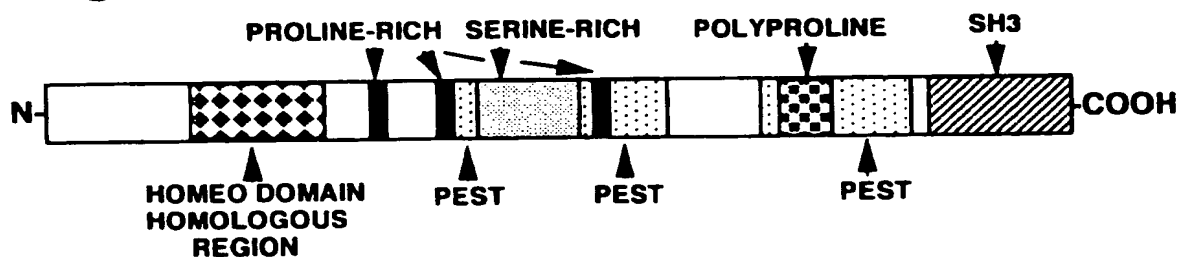
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Fig. 1A

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1  MSCRCWISRHPSEYEGWNLQSIIFHKQIRGVDLESTFVTKFGNCSLRLNETVDIHKEKVA 60
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121  PRTTPPTQKPPSPMSGKGTILGRHSPYRTLEPVRPPVVPNDYVPSPTRNMAPSQOSPVRT 180
181  ASVHQNRRTYSSSSGSSGGSEHPSSRSSSRENSGSGSVGVPIAVPTSPSPSVFPGHPVQFYS 240
241  MNRPASRHTPPTIGGSLPYRRPPSITSQTSLQNMNGGPFYSONPVSDTPPPPPVEEPV 300
301  FDESPPPPPPPEDYEEEEAAVVEYSOPYAEEDPPWAPRSYLEKVVAIYDYTKDKEDELSF 360
361  QEGAIIVVIKKNDDGWYEGVMNGVTGLFPGNYVESIMHYSE 401

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Fig. 1B**Fig. 1C**

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ftz KR.TRQTYTRYQTLELEKEFHFNRYITRRRRIDIANALSLSERQIKIWFQNRHM
hxa3 KR.GRTAYTRPQLVELEKEFHFNRYLMRPRRVEMANLLNLTERQIKIWFQNRHM
hxa4 KR.SRTAYTRQQVLELEKEFHFNRYLTRRRRIEIAHTLCLSERQVKIWFQNRHM
hxd3 KR.VRTAYTSAQLVELEKEFHFNRYLCRPRRVEMANLLNLTERQIKIWFQNRHM
hxd4 KR.SRTAYTRQQVLELEKEFHFNRYLTRRRRIEIAHTLCLSERQIKIWFQNRHM
hxi39 KR.QRTAYTRNQVLELEKEFHFNRYLTRRRRIEIAHTLCLSERQVKIWFQNRHM
zen KR.SRTAFSSLQIELEREFHLNRYLTRRRRIEISQRLALTERQVKIWFQNRHM
abi-2 KNTSRTHKIIAPAN.LERP.V...RYI.R.KPIDYT.ILDDIGHGVKVSTQNMKGGL
cons. K RT LEK F RYL R R IE L R IKIWFQNRHM
R I K VD H V V K

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Fig. 1D

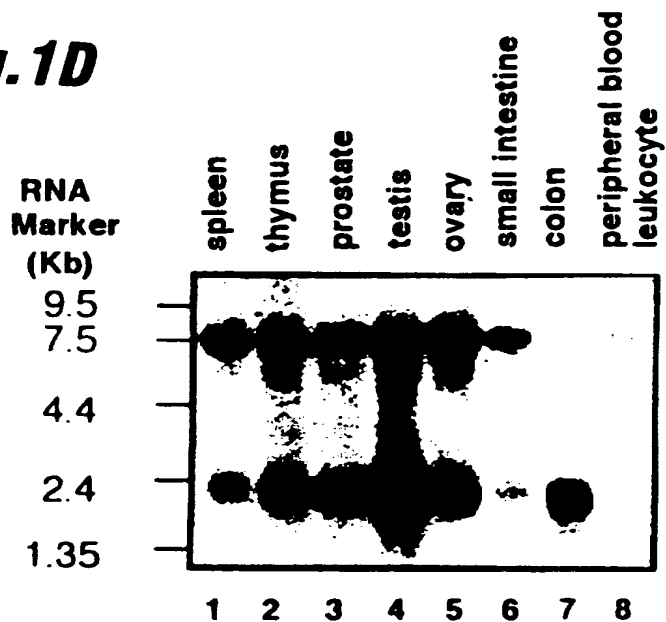
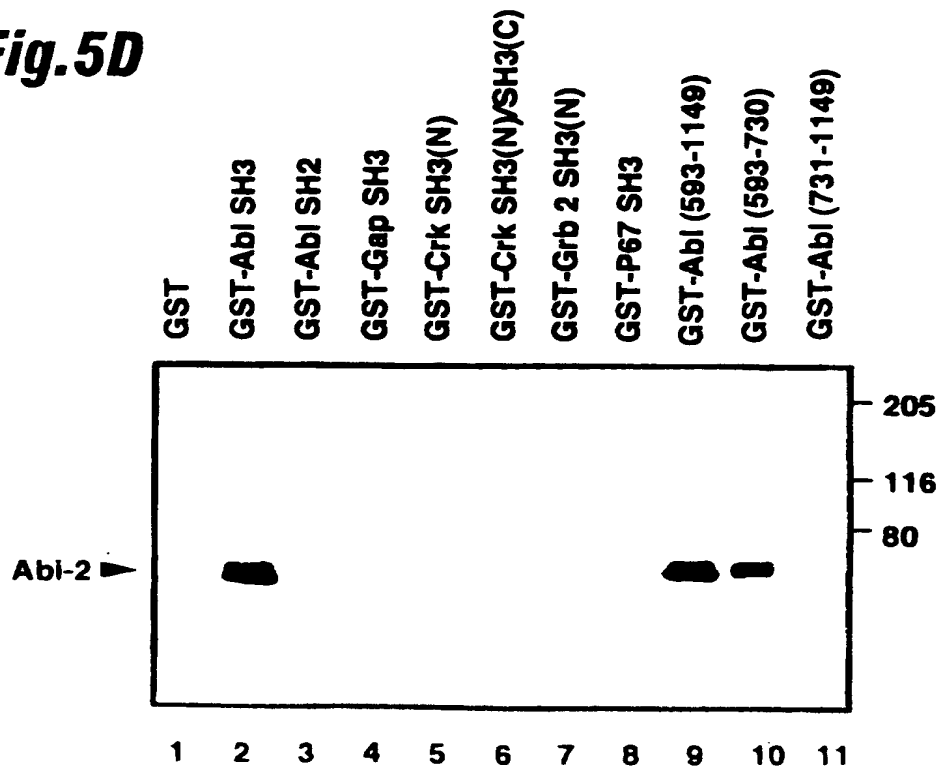


Fig. 5D



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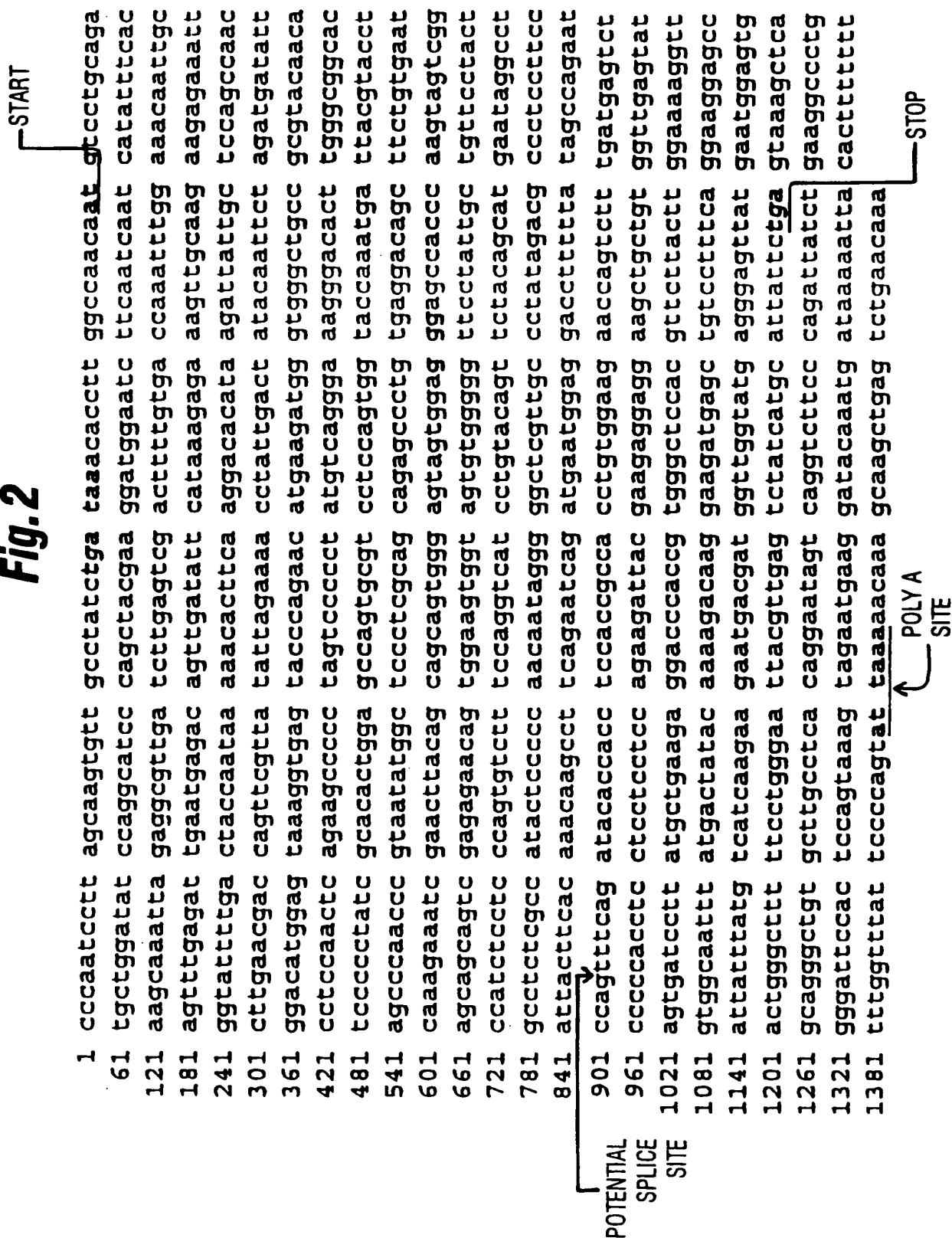
Fig. 2

Fig. 3A

In Vitro Translation

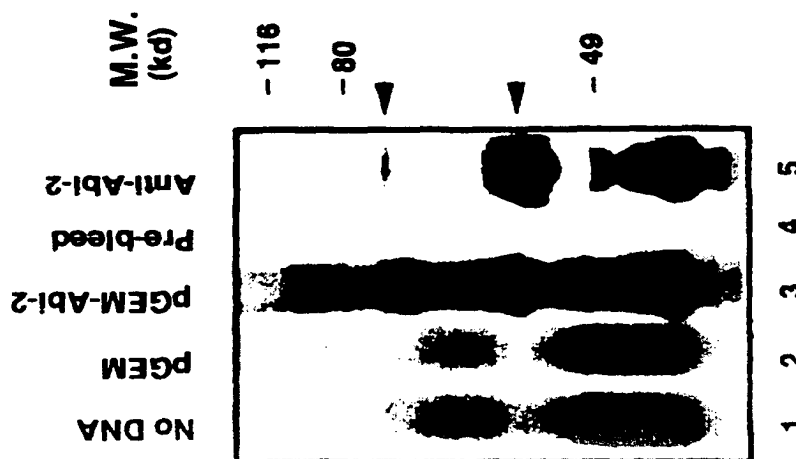


Fig. 3B

In Vivo Expression

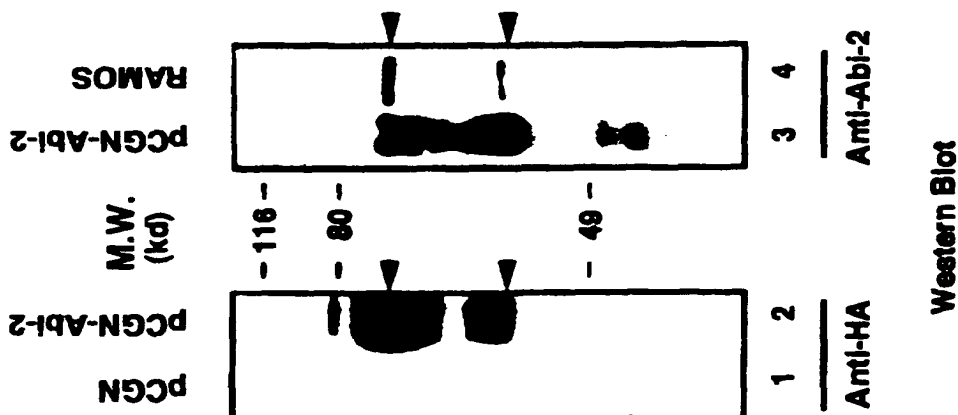


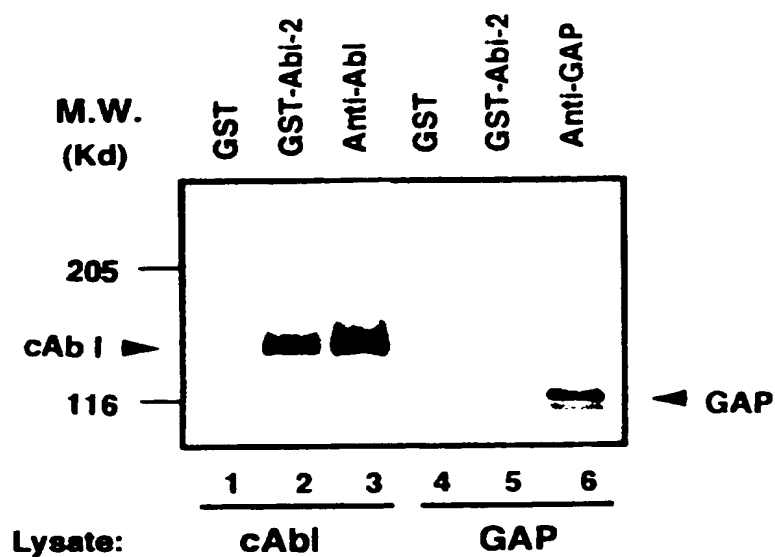
Fig. 3C

Subcellular Localization

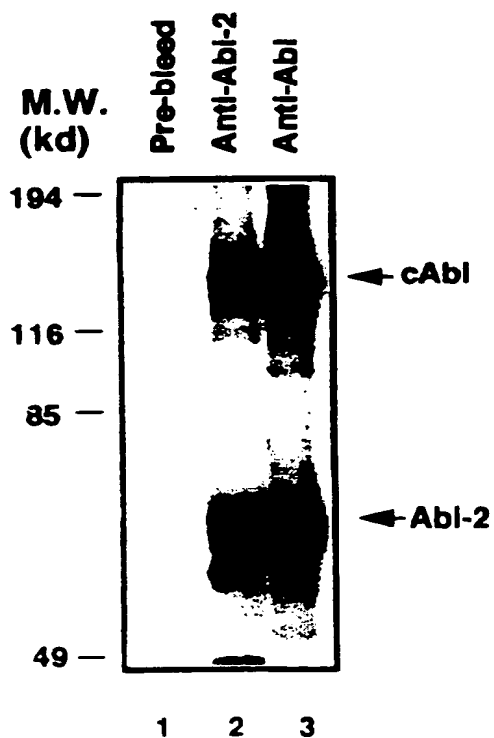


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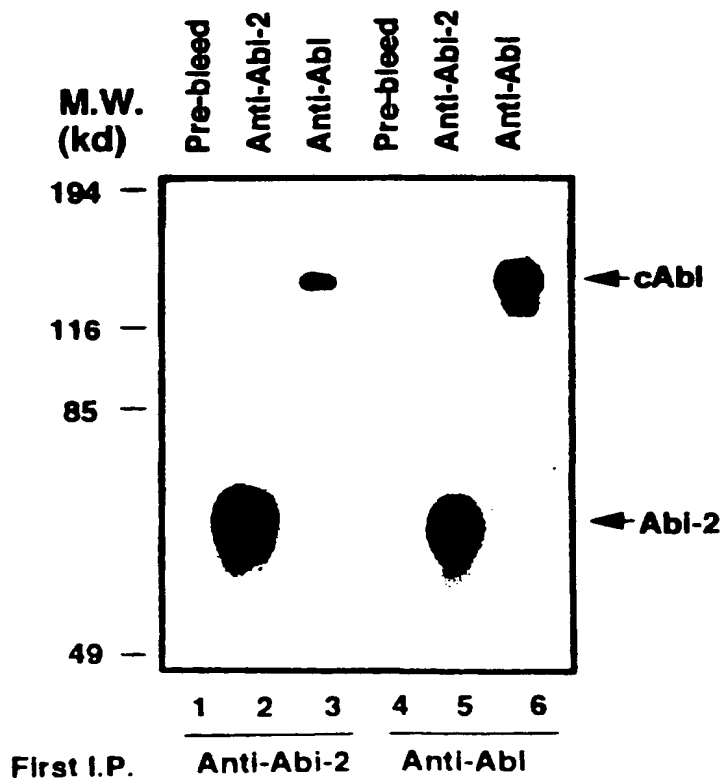
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Fig.4A**Fig.4B**

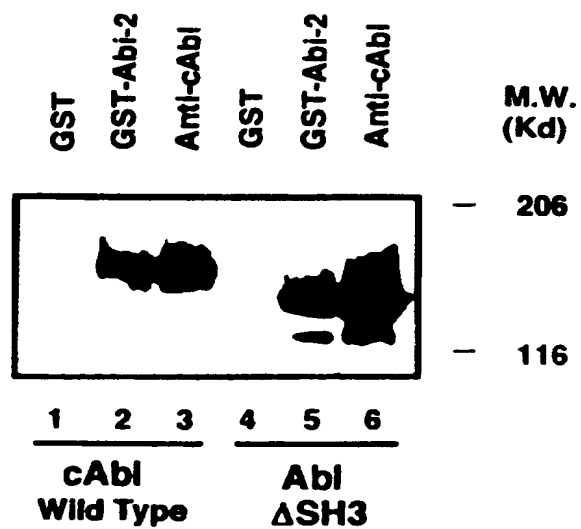
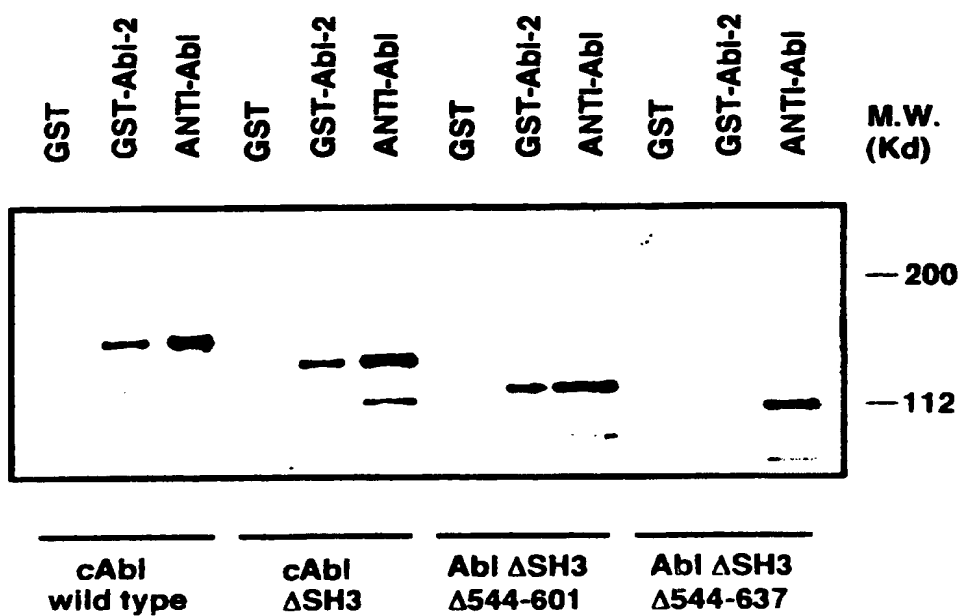
First cycle I.P.

**Fig.4C**

Second cycle I.P.

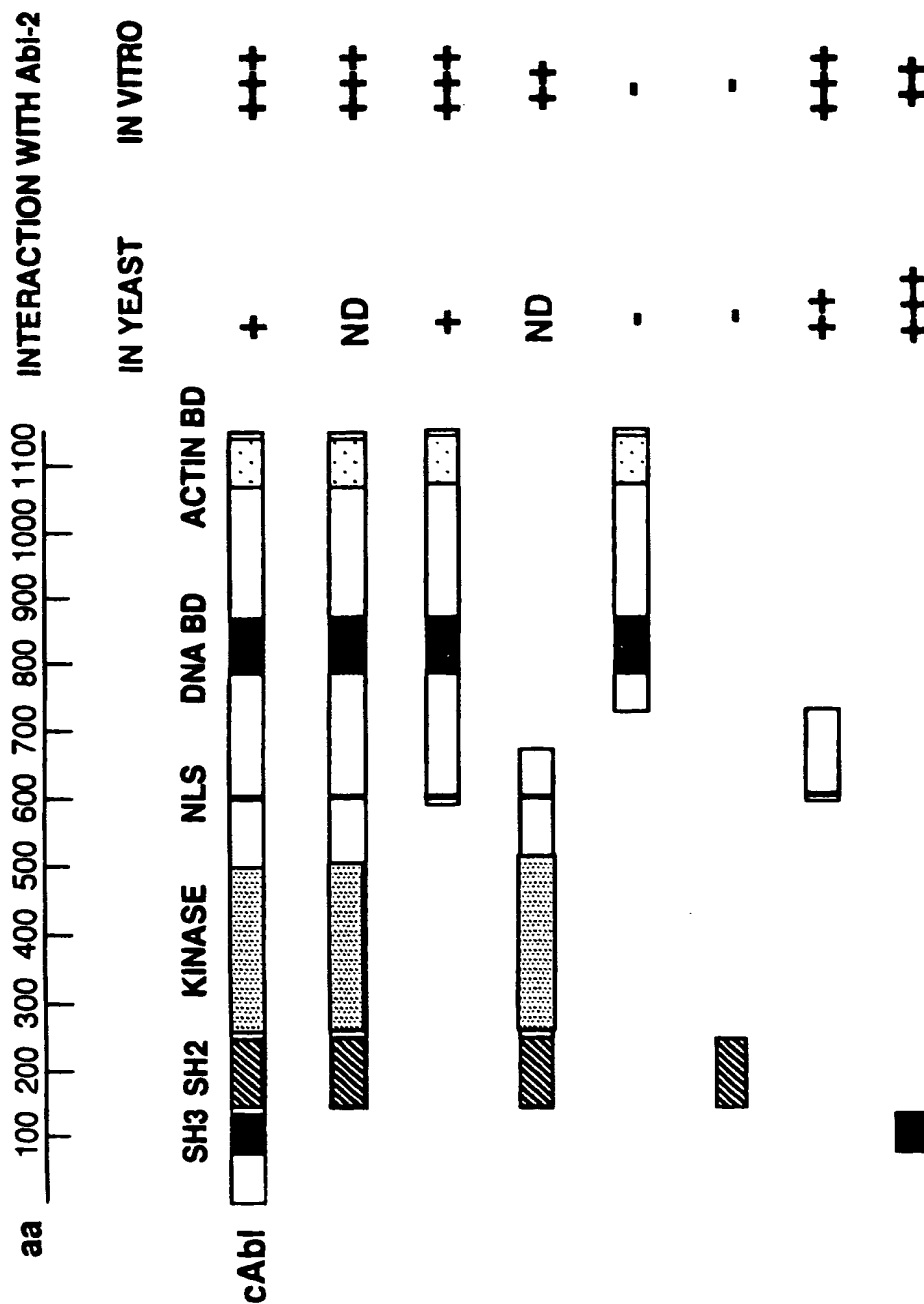


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Fig.5A**Fig.5C**

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Fig. 5B



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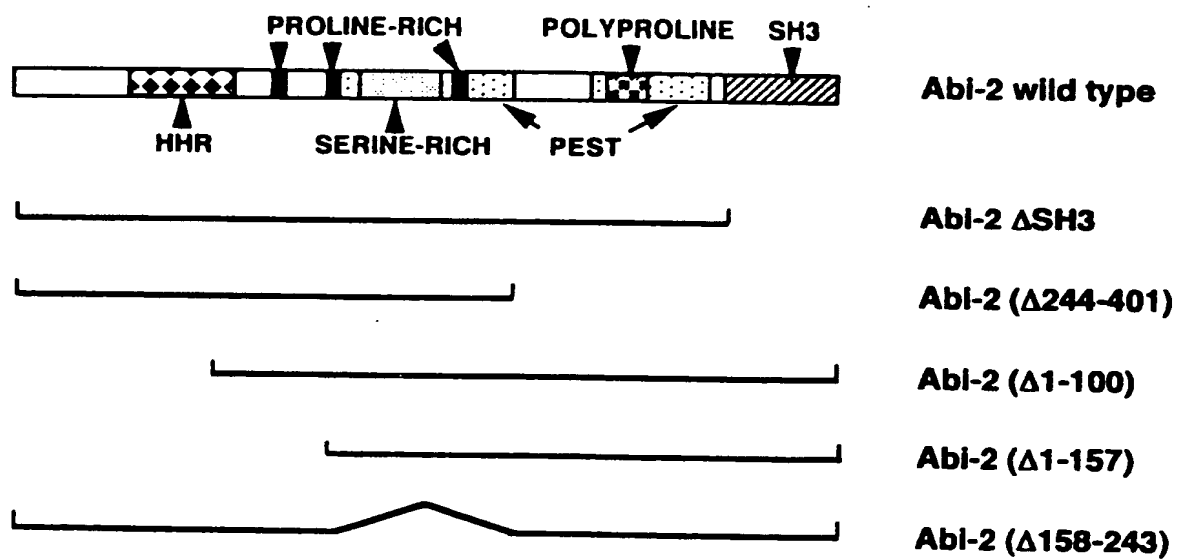
Fig. 6A

Fig. 6C

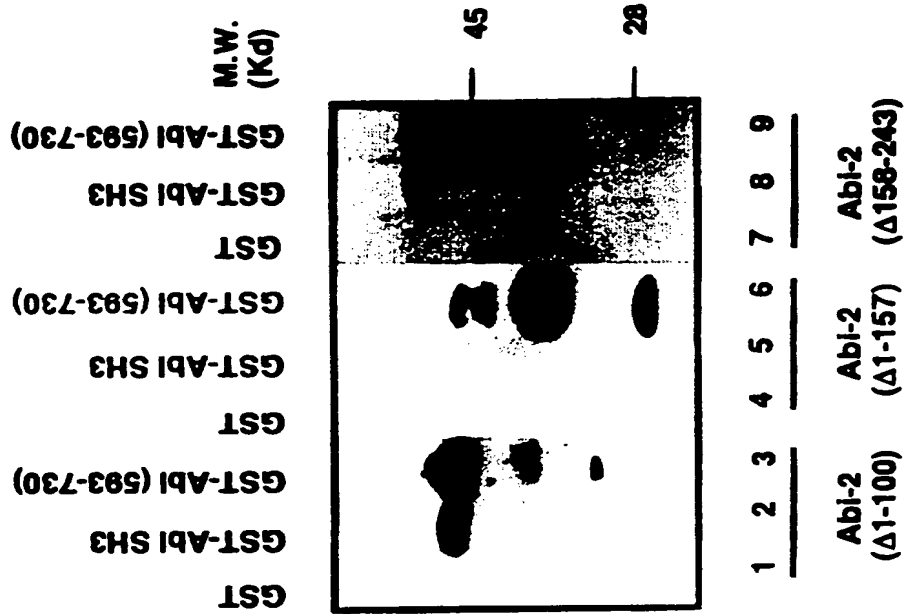
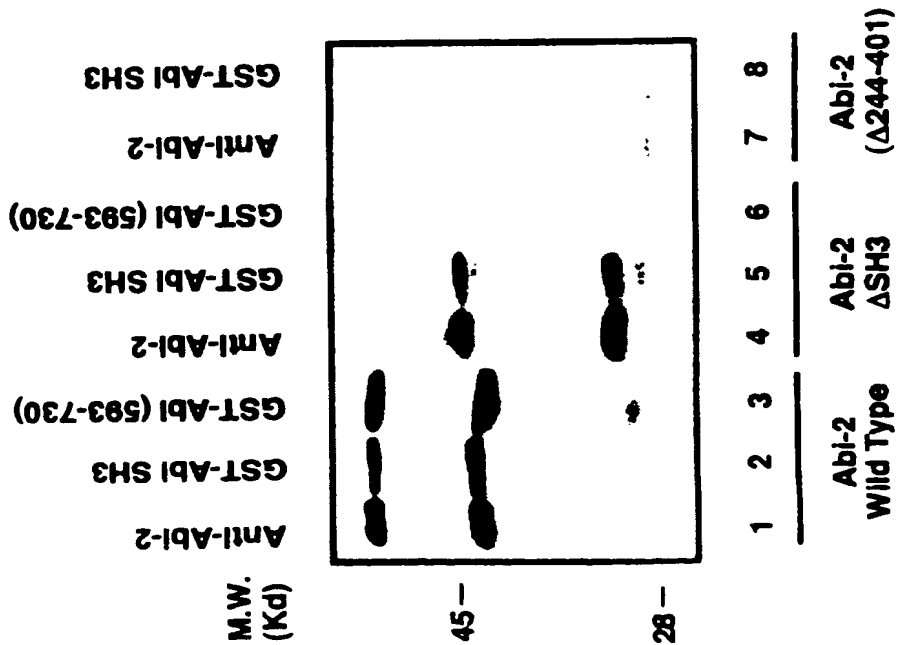
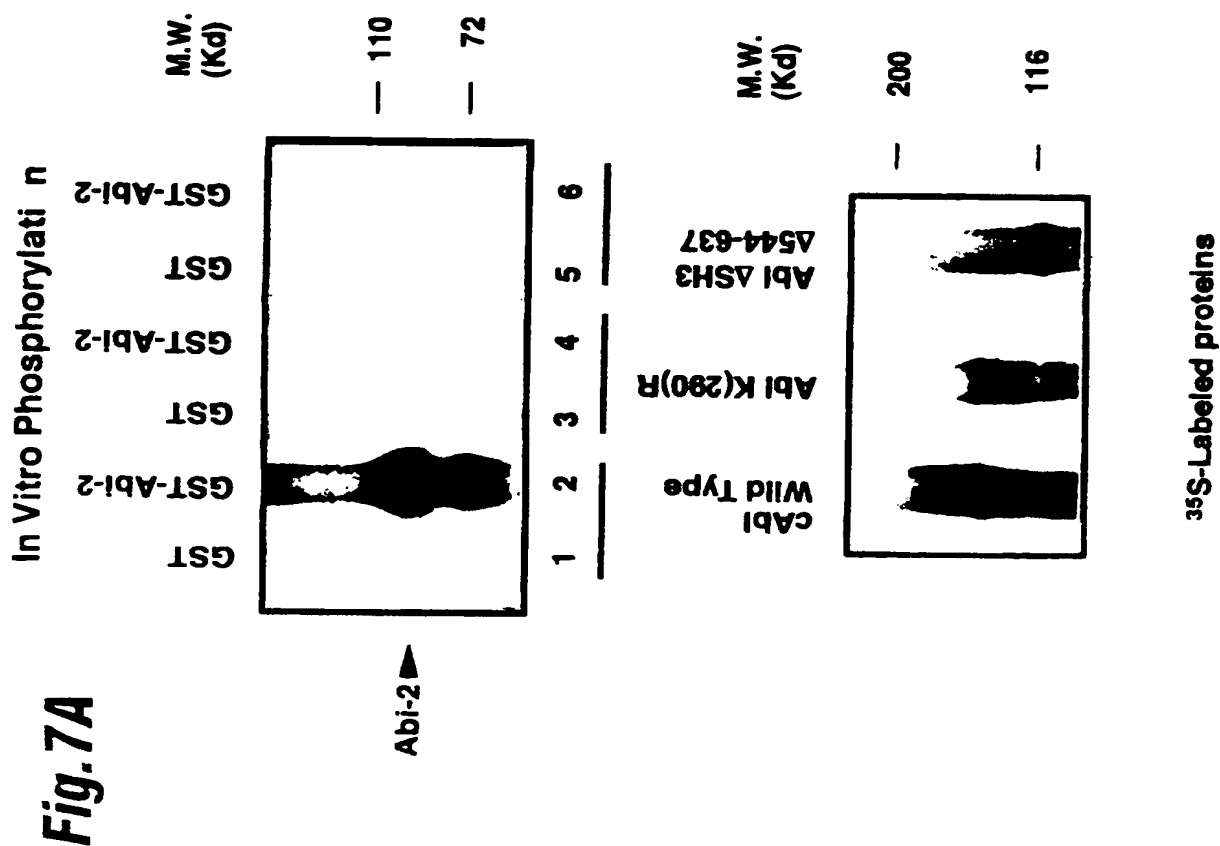
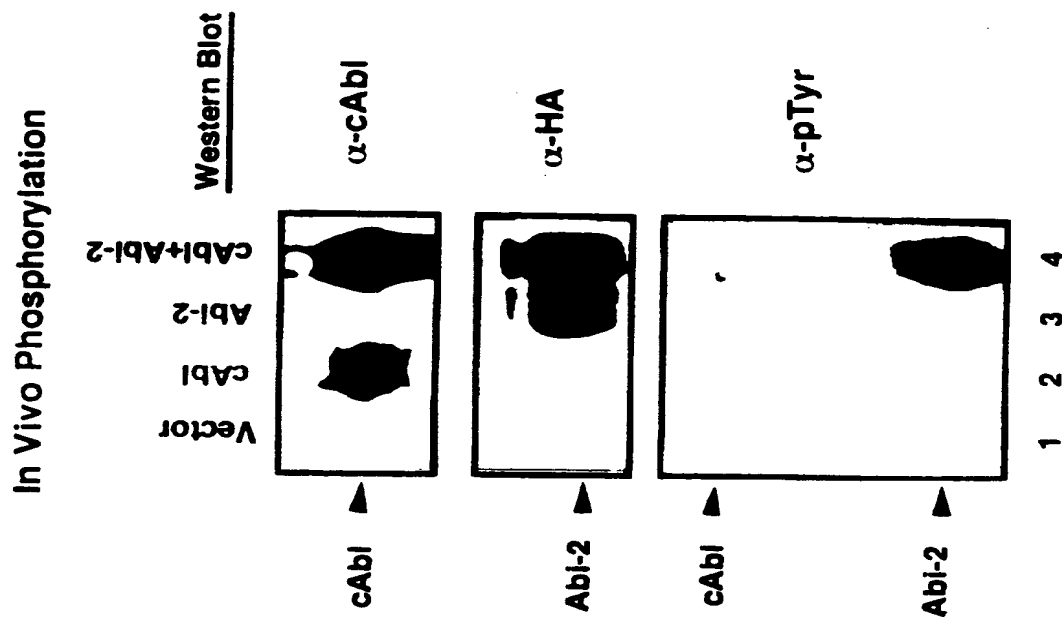


Fig. 6B



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Fig. 7B



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Fig. 8B

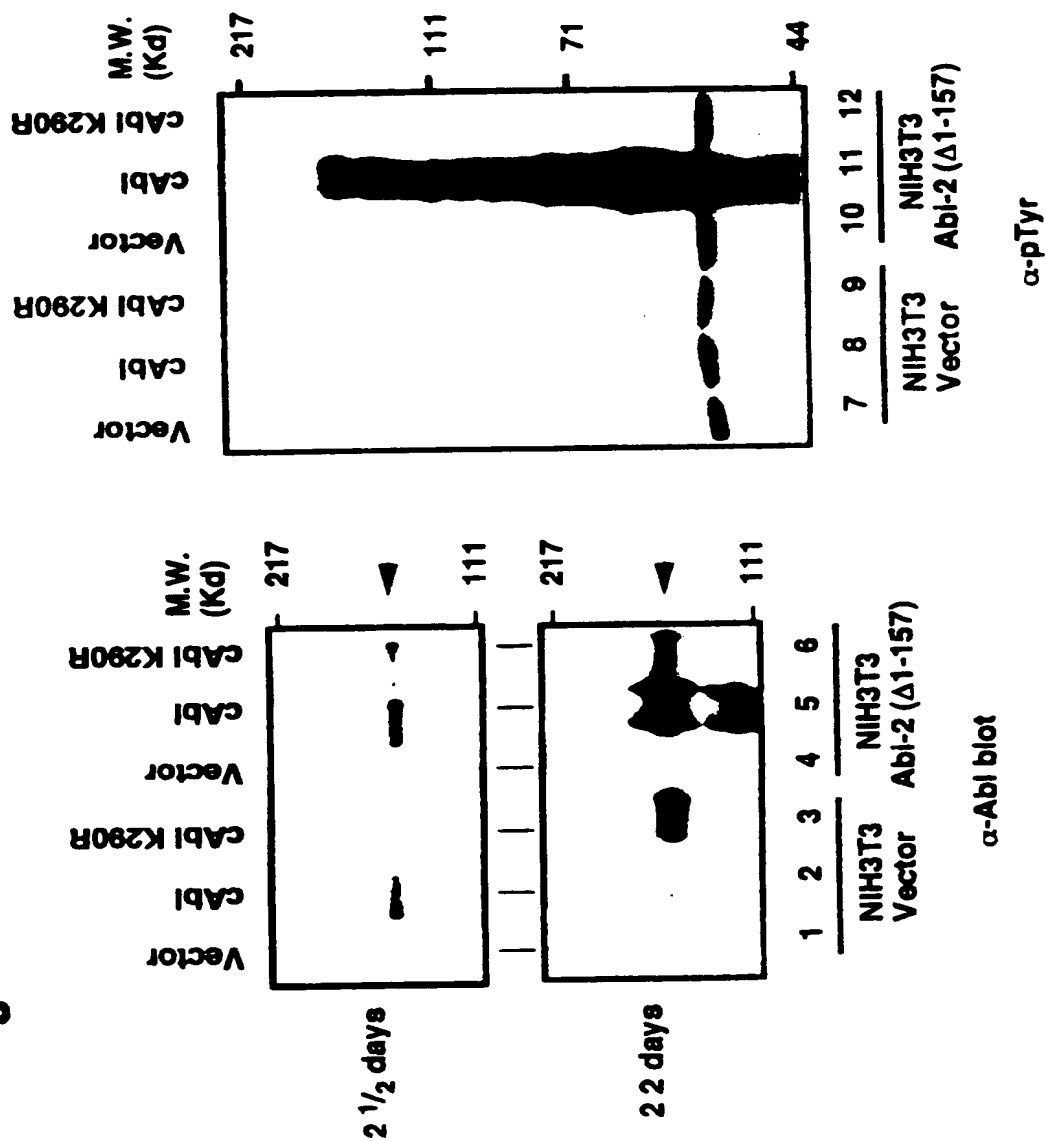
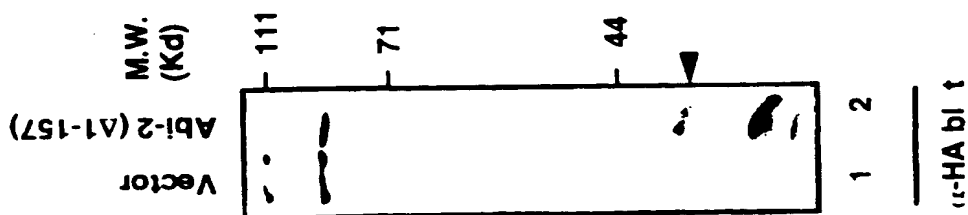


Fig. 8A



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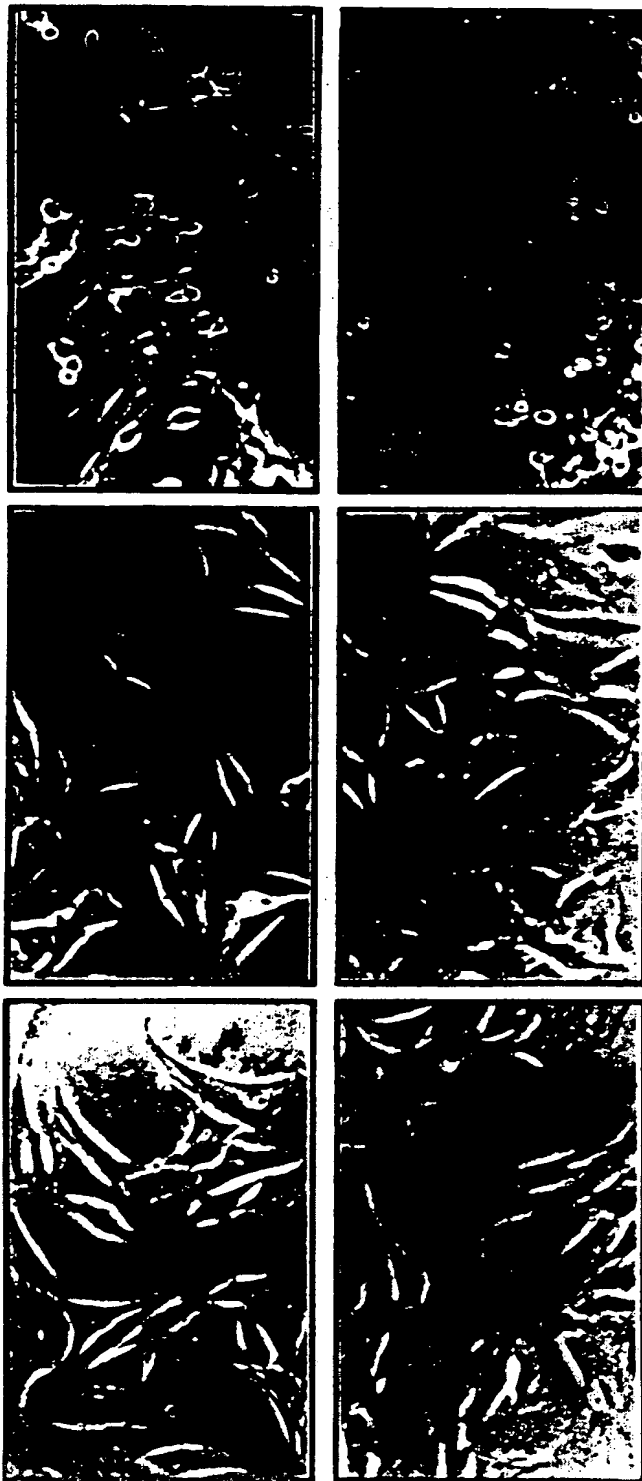


Fig. 8C

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Fig. 8D

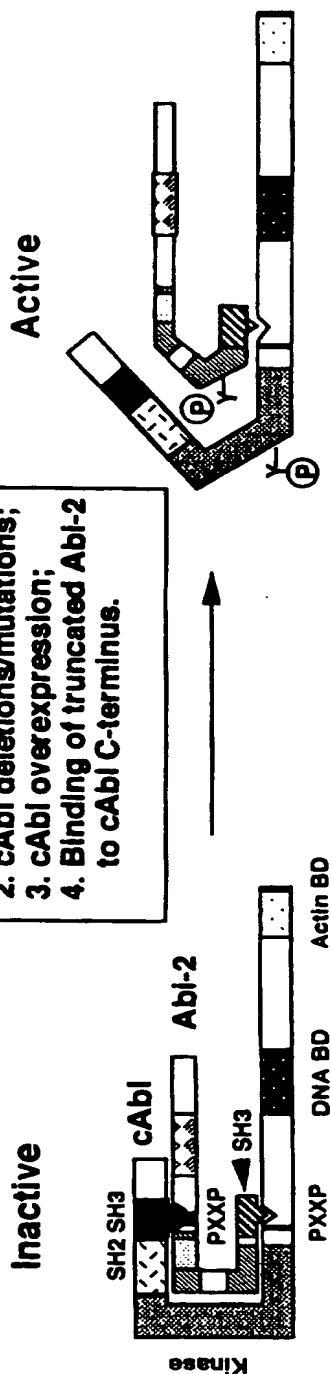
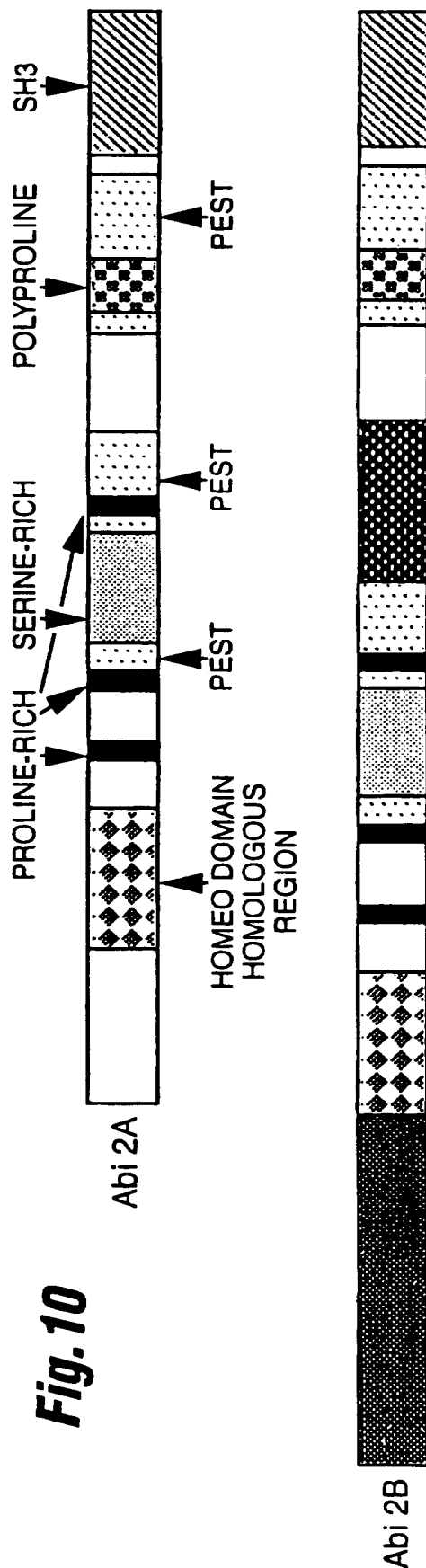
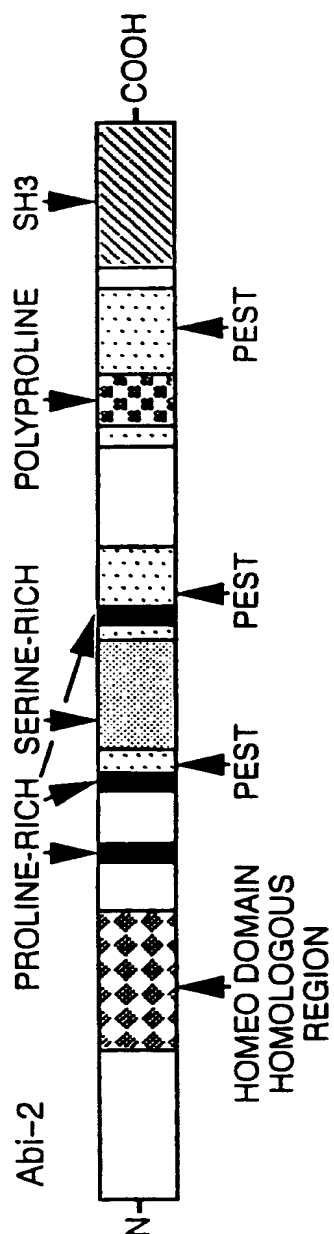
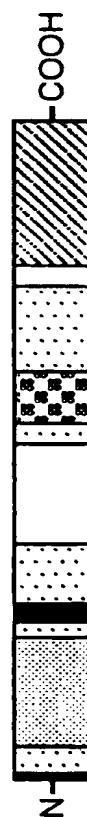


Fig. 10



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Abi-2 $\Delta 1-100$ Abi-2 $\Delta 1-157$ **Fig. 9A**

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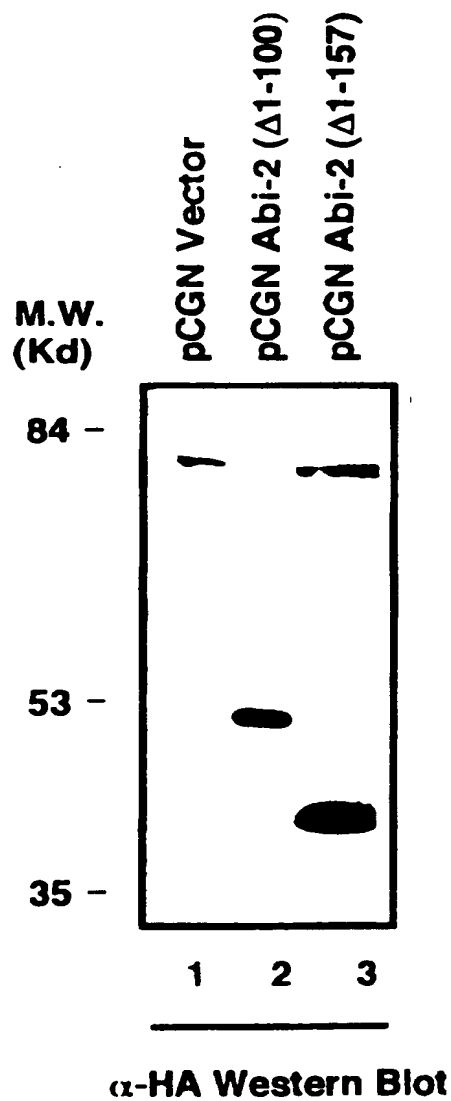
Fig.9B

Fig. 9C

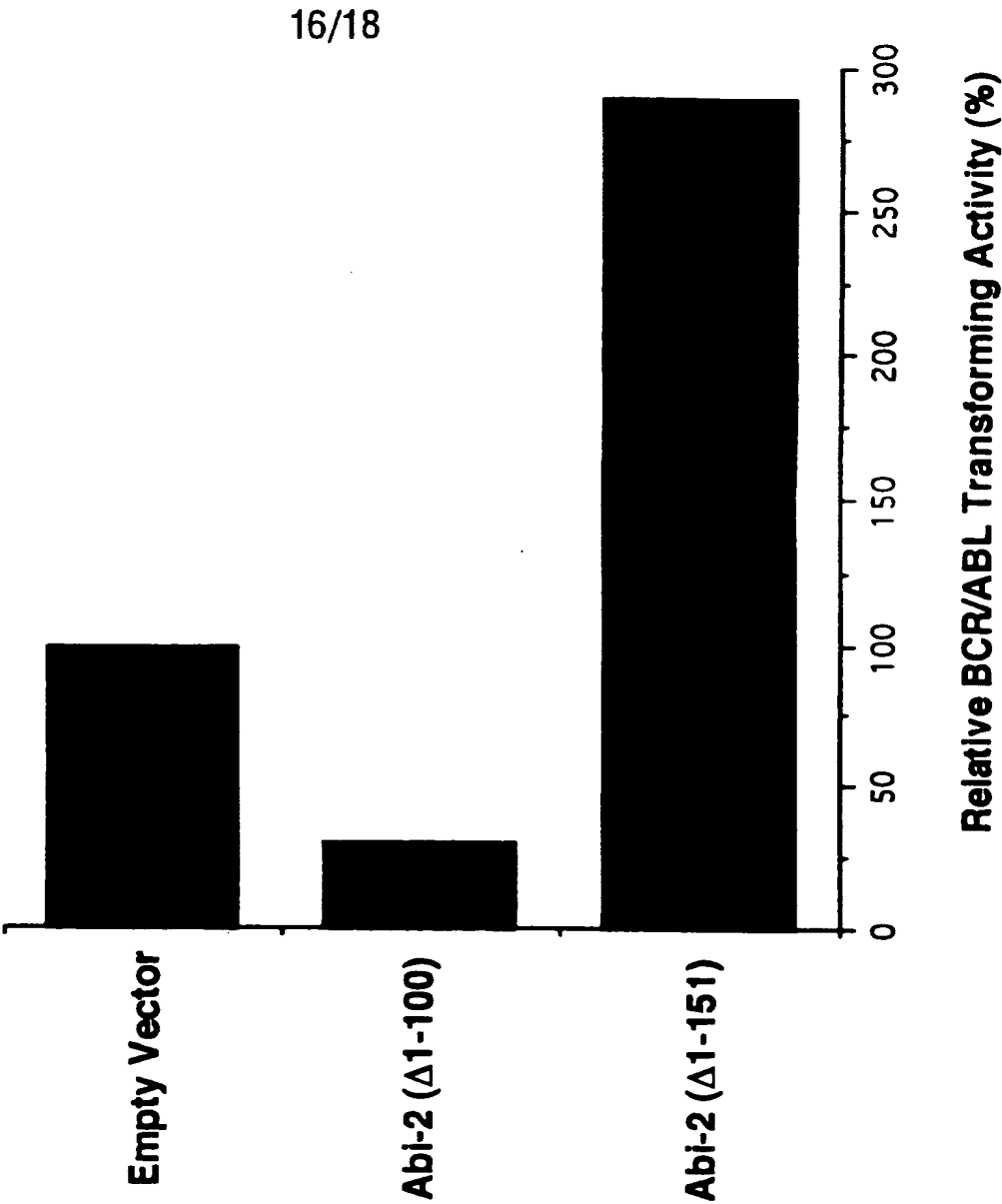
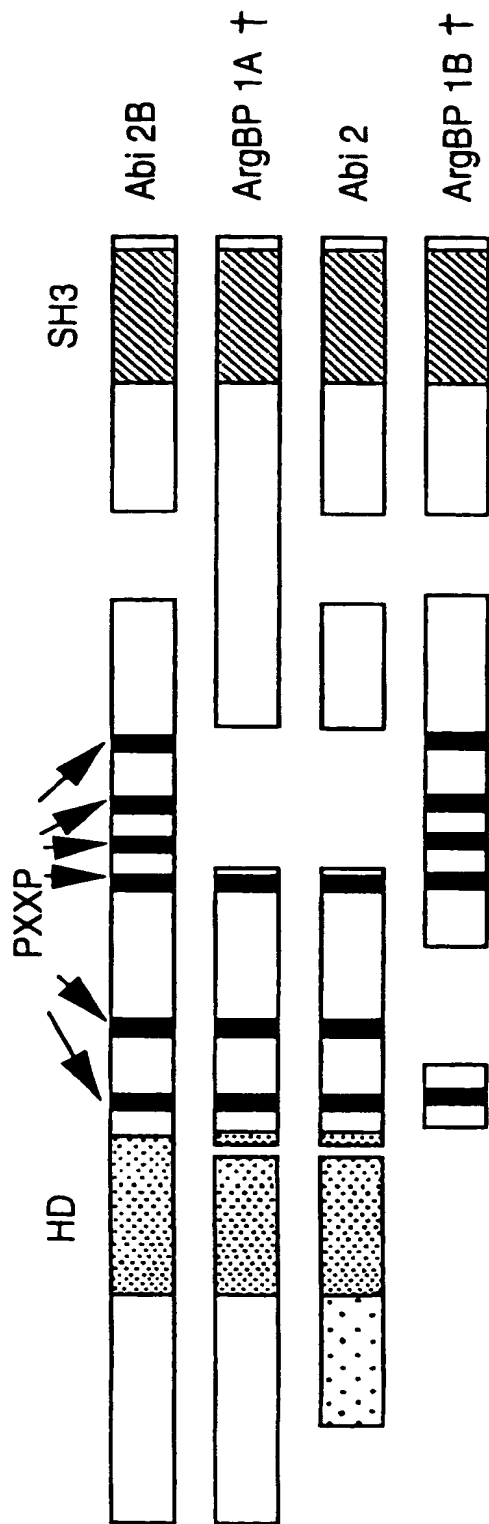


Fig. 11



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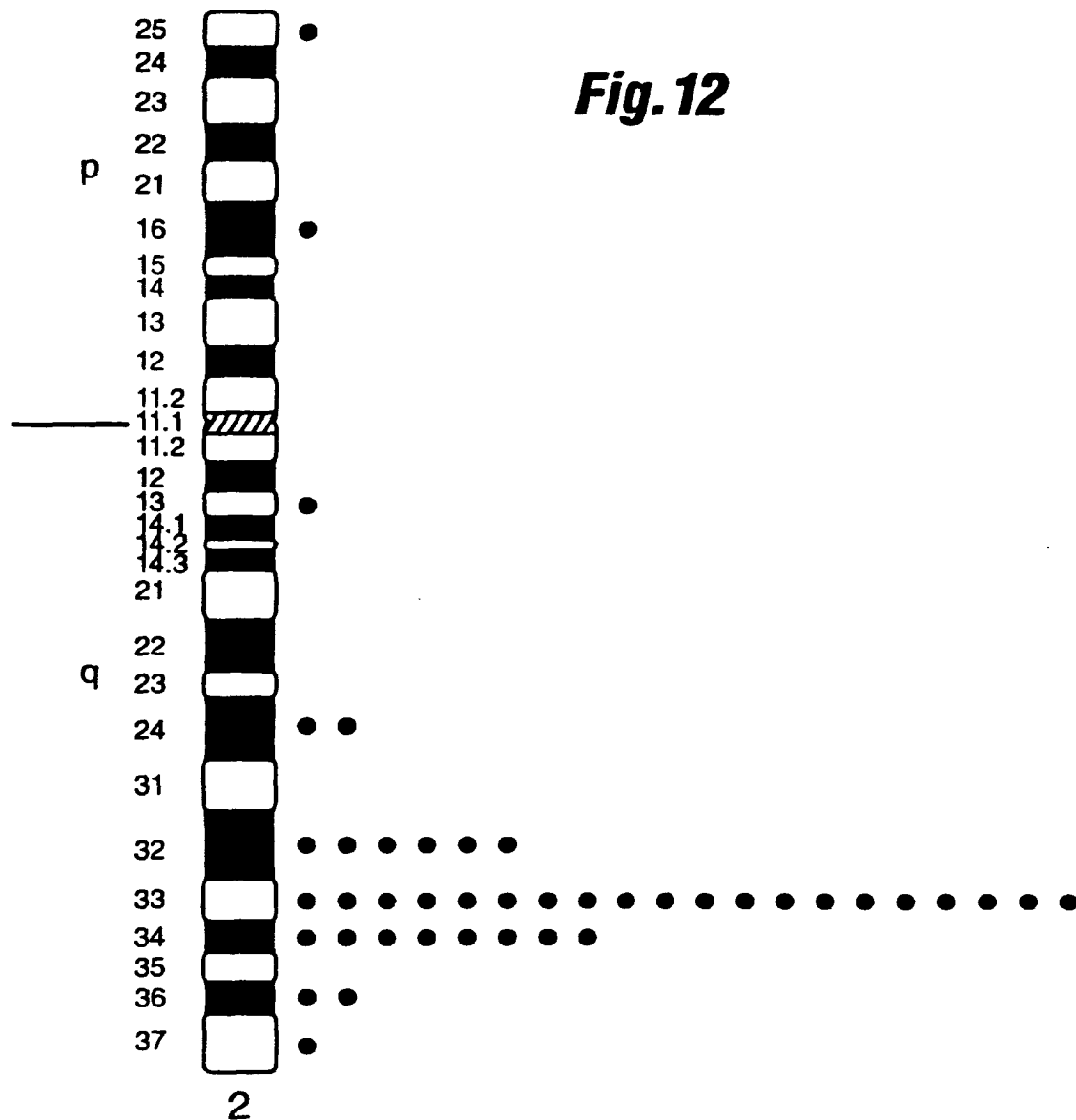
Fig. 13

Correlation of Human Sequences Detected by Abi-2 cDNA with Human Chromosomes in Rodent X Human Somatic Cell Hybrids																									
Hybridization/ Chromosome	Human chromosomes																								
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	X	Y	
Concordant	+/+	3	6	4	3	4	5	4	5	0	2	1	3	3	5	6	1	6	5	4	5	3	3	0	1
	-/-	11	12	6	5	7	5	5	11	6	8	5	9	5	6	11	3	9	7	6	7	8	10	9	
Discordant	+/-	3	0	2	2	1	1	1	0	6	3	3	2	1	0	4	0	1	2	1	2	1	4	4	
	-/+	1	0	5	6	5	7	6	5	1	2	4	7	2	7	3	1	9	3	2	5	5	4	1	2
Discordant clones		4	0	7	8	6	8	7	5	7	5	7	9	3	7	3	5	9	4	4	6	7	5	5	6
Informative clones*		18	18	17	16	17	18	16	15	18	13	16	17	15	17	15	17	18	18	15	17	17	16	15	16

*Data for chromosomes involved in rearrangement of present at a frequency of 0.1 or less were excluded.

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Fig. 12



INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/17047

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : C07H 21/04; C12N 5/10, 15/11; C12P 21/02; C07K 14/475

US CL : Please See Extra Sheet.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 536/23.5, 24.5, 24.31; 435/320:1, 240.2, 252.3, 69.1, 70.3; 530/350

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, MEDLINE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	CICCHETTI et al. 3BP-1, an SH3 domain binding protein, has GAP activity for Rac and inhibits growth factor-induced membrane ruffling in fibroblasts. Embo Journal. 03 July 1995, Volume 14, pages 3127-3135, see entire document.	1, 2, 5, 10-16

☐

Further documents are listed in the continuation of Box C.

☐

See patent family annex.

<p>* Special categories of cited documents:</p>		<p>* T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p>	
* A*	document defining the general state of the art which is not considered to be of particular relevance	* X*	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
* E*	earlier document published on or after the international filing date	* Y*	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
* L*	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	* G*	document member of the same patent family
* O*	document referring to an oral disclosure, use, exhibition or other means		
* P*	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search

Date of mailing of the international search report

03 FEB 1997

Name and mailing address of the ISA/US
Commissioner of Patents and Trademarks
Box PCT
Washington, D.C. 20231

Authorized officer
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Telephone No. (703) 203-0196

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/17047

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1-17, 24, 25

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/17047

A. CLASSIFICATION OF SUBJECT MATTER:
US CL :

536/23.5, 24.5, 24.31; 435/320.1, 240.2, 252.3, 69.1, 70.3; 530/350